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SECTION MEETINGS

CLEVELAND	
Western Reserve University	February 11, 1949
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16931

Production of Acute Pulmonary Edema by Ammonium Salts.*

Harold Koenig,† and Ruth Koenig. (Introduced by W. F. Windle.)
From Department of Anatomy, School of Medicine, University of Pennsylvania, Philadelphia.

During the investigation of another problem,[1] in which guinea pigs were administered ammonium chloride by gavage, small overdoses of this chemical were found to result in pulmonary edema after a short interval.

In guinea pigs so treated, acute pulmonary edema appeared to be a consistent cause of death.[2,3] It was therefore considered worthwhile to experiment further with the thought of utilizing ammonium salts as a tool for a subsequent investigation of the pathogenesis of acute pulmonary edema.

Methods. Guinea pigs and white rats of both sexes were principally used. Six cats and 5 rabbits also were studied. Early in these studies, complete autopsies were done but when it became apparent that changes were limited to the chest, autopsy studies were restricted to the thoracic viscera.

Microscopic studies were carried out on all lungs in which the pathological diagnosis was in doubt, and also in a series of animals in which it was desired to study the histological changes of the grossly abnormal lungs. The lungs were fixed in one of 3 ways: by vascular perfusion[4] of 10% formalin in isotonic saline solution with 2.4% gum acacia added; by intratracheal injection of 10% formalin in isotonic saline solution; or by ligating and excising a pulmonary lobe, immersing it in 10% formalin in isotonic saline, and agitating it constantly on a shaking device for 24-48 hours. Immersion fixation of an entire lobe gave the best results for the demonstration of edema exudate. All lung sections were stained with hematoxylin and eosin.

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* This work was aided by a grant from the Baxter Laboratories, Inc., administered by Professor W. F. Windle.

† Presented to the Graduate School of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[1] Windle, W. F., Koenig, H., and Jensen, A. V., *Arch. Neurol. and Psychiat.*, 1946, **56**, 428.

[2] Koenig, H., and Koenig, R., *Fed. Proc.*, 1948, **7**, 67.

[3] Koenig, H., and Koenig, R., *Anat. Rec.*, 1948, **100**, 52.

A 6% solution of ammonium chloride in water (weight in volume) was used at all times with the exception of the animals administered other salts of ammonium to determine the specificity of the cation with regard to the lung changes. Control animals were treated like the experimental animals except for the administration of ammonium chloride. These animals were also anesthetized with pentobarbital ("Veterinary Nembutal") just before removal of the lungs. Further details of procedure will be given in the appropriate sections to follow.

Results. A. Dosage and routes of administration. Guinea pig. Most of our experience has been with this animal. We have seen typical pulmonary alterations with ammonium chloride given by the gavage, subcutaneous, intramuscular, intraperitoneal and intravenous routes of administration. The dose of ammonium chloride depended upon its mode of introduction. The slower the rate of absorption, the larger the dose had to be. Thus, a dose which would produce acute pulmonary edema by gavage would, when injected into the peritoneal cavity, usually kill the animal before sufficient time had elapsed for the evolution of these changes. Ammonium chloride could be successfully injected into the vein only by slow drip. Another factor of importance was the weight of the animal; the usual direct relationship between body weight and dosage appeared to apply.

The gavage and intraperitoneal routes were utilized almost exclusively because the results were more dependable. By stomach tube, the effective dose was 0.09 to 0.12 g/100 g of body weight; intraperitoneally, it was 0.05 to 0.07 g/100 g of body weight. Thirty of the 40 experimental guinea pigs succumbed to acute pulmonary edema. Wherever lung changes failed to appear, it could be attributed to one of two causes. If the amount of ammonium chloride was too great, or if it was too rapidly absorbed, death resulted from cardiac failure, or more frequently from respiratory failure, before the appearance of lung changes. On the other hand, if the dose was inadequate, or if absorption appeared retarded, the lung changes were not seen and other manifestations of toxicity were often completely absent. Our experience suggested that a definite concentration of the ammonium ion has to be attained and maintained in the blood for a period of at least 10 or 15 minutes for the development of pulmonary edema.

Rat. Ammonium chloride solution was administered to this animal by stomach tube and by intraperitoneal injection. The latter route proved more convenient and reliable. The effective dose for the intraperitoneal route was 0.04 g/100 g (0.66 cc of a 6% solution). Administration resulted in acute pulmonary edema in all of 10 rats.

Cat. The cat proved to be more resistant to the pulmonary edema produced by ammonium chloride than the guinea pig or the rat. Vomiting and salivation were a common occurrence with toxic doses of ammonium chloride given intraperitoneally or by gavage, but care was taken to prevent aspiration. In later experiments, the trachea was cannulated following local infiltration with 1% procaine in order to circumvent these difficulties. Of the 6 cats used, pulmonary edema occurred in 3 animals. The animals in the latter group weighed 1950-2750 g each. Two were given 35 cc of a 6% solution of ammonium chloride intraperitoneally. A third was given 69 cc of a 6% solution of ammonium chloride intraperitoneally in 4 divided doses over a period of 2 hours.

Rabbit. In none of 5 animals did acute pulmonary edema occur after ammonium chloride was given intraperitoneally or by gavage in doses great enough to cause toxic symptoms culminating in death.

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[4] Koenig, H., Groat, R. A., and Windle, W. F., *Stain Technology*, 1945, **20**, 13.

B. Specificity of the ammonium ion. In order to determine the specificity of the ammonium moiety of ammonium chloride in this phenomenon, 6 guinea pigs, each weighing about 350 g, were used. One was given 6 cc of 20% ammonium nitrate, a second was given 7 cc of 6.4% ammonium acetate, a third 7 cc of 10% ammonium bromide, a fourth 7 cc of 6% ammonium chloride, and a fifth 7 cc of 7.4% ammonium sulfate. The 4 latter solutions were roughly equivalent with respect to the concentration of ammonium. A sixth animal was given 7 cc of water to serve as a control. Gavage administration was used in these experiments. The first 5 animals all succumbed to acute pulmonary edema. The lungs of the control were normal.

Guinea pigs given the standard dose of ammonium chloride by gavage showed a blood pH of 6.8 to 7.2, 15 to 30 minutes later. Two mechanisms are involved in the production of this acidosis, acid hydrolysis of ammonium salts, and the conversion of ammonia into urea. The first mechanism does not operate with an ammonium salt of a weak acid, such as ammonium acetate. However, guinea pigs and cats rendered even more severely acidotic by gavage administration of a sodium lactate buffer, or a dilute solution of hydrochloric acid, never showed pulmonary edema. It is evident, therefore, that the ammonium ion rather than the acidosis is the primary factor in the pathogenesis of this form of lung edema.

C. Syndrome of ammonium intoxication. In the guinea pig and rat, the characteristic sequence of events transpired in 15 to 60 minutes, taking less than 30 minutes in most of the animals. A considerable increase in rate and depth of respiration occurred several minutes after the administration of ammonium chloride. Weakness and difficulty in locomotion soon appeared; at about the same time, hyperexcitability for tactile, auditory and painful stimuli was noted. Shortly thereafter, muscle fasciculations appeared over most of the body and could be best seen in the tongue. These usually preceded the onset of one or more generalized tonic convulsions. Occasionally the convulsions appeared before the fasciculations. Coma quickly supervened, the corneal reflex was absent and no stimulus however great would bring about a response. The respirations were by this time greatly reduced in frequency and were deep and gasping in character. Stridor was frequently heard. A serosanguineous, or rarely a serous, fluid exuded from the nostrils or mouth before death. Occasionally, no fasciculations or convulsions occurred, or perhaps only a terminal convulsion; yet acute pulmonary edema was present at autopsy.

The above description held also for the cat, with several exceptions. The dose required was greater and the time interval before lung edema appeared was longer, varying from one to 3 hours. Sialorrhea usually occurred and vomiting was common. This animal appeared to be on the whole more resistant to ammonium pulmonary edema than were the rodents. Another notable difference was the character of the edema fluid issuing from the upper respiratory tract. In the cat, the fluid was never blood-tinged, whereas, in the rat and guinea pig, blood-tinged edema fluid was the rule, with very few exceptions.

The effects of toxic doses of ammonium would appear to be chiefly on the central nervous system, at least until pulmonary edema supervenes. They were manifested by muscle fasciculations, tonic convulsions and

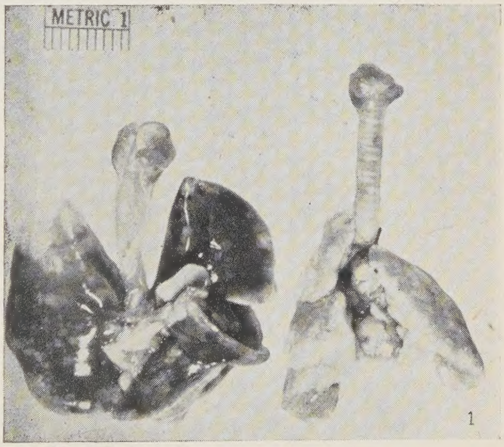
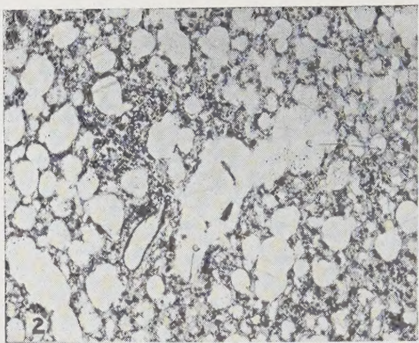
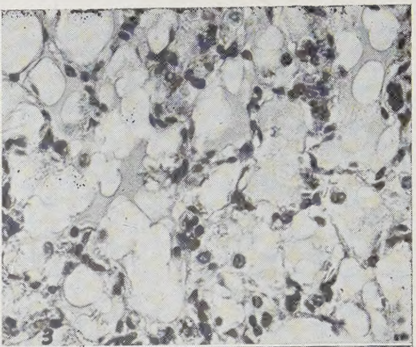
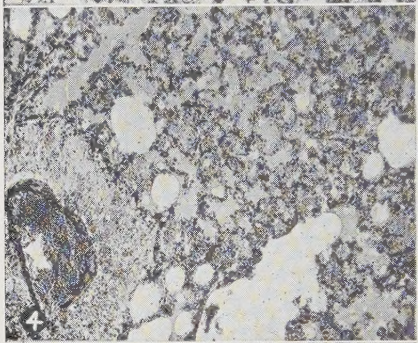
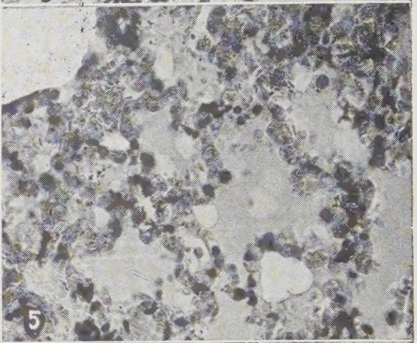


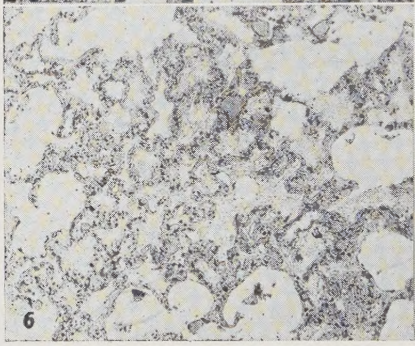
FIG. 1.
Photograph of gross specimens. On the left are the trachea and lungs from a rat that died 20 minutes after an intraperitoneal injection of ammonium chloride. The lungs are swollen and present pleural surfaces which are discolored by areas of hemorrhage and congestion. At the right are the trachea and lungs from a control rat.











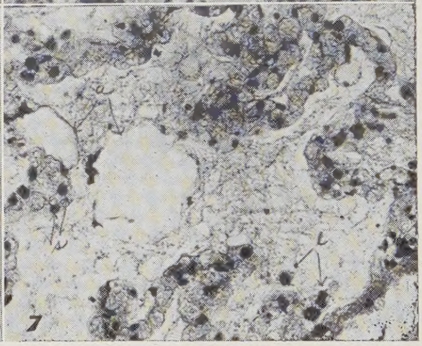


FIG. 2. Photomicrograph of a typical area from a section of lung from a guinea pig which succumbed to acute pulmonary edema 30 minutes after gavage administration of ammonium chloride. Many of the alveoli are filled with a homogeneous precipitate of edema fluid (e). × 64.

FIG. 3. Photomicrograph, at higher magnification, of part of the area shown in Fig. 2 illustrating the homogeneous edema exudate which contains many air bubbles. Hemorrhage is not a prominent feature here, but occasional small groups of extravasated red blood cells are present in the alveoli. × 263.

FIG. 4. Photomicrograph illustrating a typical area of a section of lung from rat which died 25 minutes after an intraperitoneal injection of ammonium chloride. Most of the alveoli in this field are filled with a homogeneous eosinophilic exudate in which air bubbles and occasional blood cells are included. A branch of the pulmonary artery in the lower left hand field is surrounded by a wide "collar" of edema exudate containing many red blood cells. × 64.

FIG. 5. Photomicrograph at higher magnification of a portion of the field shown in Fig. 4. The edema exudate and the dilatation and intense congestion of the alveolar capillaries are prominent features. × 263.

FIG. 6. Photomicrograph of a section taken from the lung of a cat which died of acute pulmonary edema $2\frac{1}{2}$ hours after the intraperitoneal injection of ammonium chloride. A fibrillar exudate fills many of the alveoli. A number of small veins are seen dilated and filled with blood cells. × 64.

Fig. 7. Photomicrograph at higher magnification of part of a single alveolus in the field shown in Fig. 6. Many fibrillar strands are in the edma exudate. A small bubble (a) of air still remains in the center of the alveolus, another smaller one is at the side. Occasional macrophages (septal cells(s)), and leucocytes (l) appear in what was formerly the alveolar space. Capillaries in the alveolar septa are turgid with blood cells. × 263.

dyspnea. The fasciculations suggested repetitive discharging of primary motor neurons of the brain stem and spinal cord. The tonic convulsions indicated a greatly heightened reflex excitability of the spinal cord. The dyspnea which occurred early had a two-fold origin. Acidosis was an important factor, which probably stimulated respiration reflexly through the chemoreceptors[5] and through an increase in CO_2 tension in the blood which resulted from a depression in pH. In addition, ammonium is a direct respiratory stimulant;[6] we noted dyspnea with ammonium acetate, a salt which produces no immediate depression of blood pH.

D. Anatomical changes. Gross pathology found at necropsy was observed only in the chest and upper respiratory tract. The nasal cavity, larynx, trachea and bronchi were filled with a frothy fluid, usually blood-tinged in the guinea pig and rat, but serous in the cat. These passageways were otherwise unobstructed. Pleural fluid was never seen. The lungs were increased in size (Fig. 1), collapse being prevented by the fluid in the lung parenchyma. The pleural surface of the lungs was always discolored; in some animals there was only a petechial distribution of deep red spots, in others a massive hemorrhagic involvement, but an appearance intermediate between these extremes was more common. The changes showed no predilection for lobes or parts of lobes. On section, the lung was deep red in many places and a frothy serosanguineous fluid could be readily expressed. Congestion of blood vessels and parenchyma was marked. The right atrium, right ventricle and the venae cavae entering the heart were usually moderately dilated, but the left ventricle and left atrium appeared normal.

Histological alterations were limited to the lung and the brain.[‡] By far the most important change was the massive pulmonary edema with variable degrees of hemorrhage. The alveolar spaces and smaller air passages were filled with a precipitated pale eosinophilic material in which air bubbles and red blood cells were often included. This edema exudate was usually homogeneous in the guinea pig (Figs. 2 and 3) and rat (Figs. 4 and 5) when the lung was fixed by total immersion, but was distinctly fibrillar in the cat. Interstitial edema was not a prominent finding except in the rat where periarterial "collaring" or "cuffing" of edema exudate was always present. Hemorrhage into the alveoli and the interstitial tissues was always present, but varied greatly in extent and degree. Vascular congestion was intense and involved not only the larger vessels, but also the capillaries. In the cat (Fig. 6 and 7) it was not unusual to find alveolar capillaries that were seven or eight red blood cell diameters in width and filled with densely packed blood cells.

Discussion. The lung changes produced by toxic doses of ammonium salts appear to have escaped notice. Underhill and Kapsinow[7] determined the minimum lethal dose for a series of ammonium salts in white rats, producing death within three hours. They concluded that the toxicity of these compounds, with a few exceptions, varied directly with the ammonium content of the compound. However, these investigators did not report the presence of pulmonary edema, nor did they state the cause of death in the animals studied. Chenoweth and his colleagues[8] studied the toxicity of ammonium gluconate which was given intravenously to cats, but they did not record the presence of pulmonary edema at autopsy.

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[5] Schmidt, C. F., Macleod's Physiology in Modern Medicine, Philip Bard, C. V. Mosby Co., St. Louis, 9th edition, 1941, 561, 567.

[6] Brassfield, C. R., Hansen, E. T., and Gesell, R., *Fed. Proc.*, 1946, **5**, 10.

[‡] Pathological changes have been seen in the brain and are described elsewhere.[1]

[7] Underhill, F. P., and Kapsinow, R., *J. Biol. Chem.*, 1922, **54**, 451.

[8] Chenoweth, M. B., Civin, H., Salzman, C., Cohen, M., and Gold, H., *J. Lab. and Clin. Med.*, 1941, **26**, 1574.

It is in the clinical literature that one finds a hint of this toxic effect of ammonium salts. Fazekas[9] described 2 cases of suicide resulting from the ingestion of ammonium chloride. One woman died 10 hours afterward and autopsy revealed, among other findings, an advanced pulmonary edema. The brain changes which were described were characteristic of anoxia. The amount of ammonium chloride ingested was unknown.

Abnormal increases of ammonium in the blood are occasionally encountered in disease. McNeil and Levy[10] found that this occurred most often in conjunction with acute disease processes involving the liver. In view of the important role the liver normally plays in the transformation of ammonium to urea, this is not surprising. While there is apparently little danger of ammonium intoxication with the usual therapeutic doses of ammonium salts, the possibility exists that large doses, such as are occasionally given to cardiac patients to promote diuresis (up to 40 g a day)[11] in the presence of an hepatic insufficiency, may result in a blood concentration of ammonium great enough to cause toxic manifestations—even pulmonary edema. Such an occurrence would ordinarily be imputed to the cardiac disorder rather than the ammonium.

Summary. (1) Ammonium salts in toxic doses produced an acute pulmonary edema in the rat, guinea pig and cat. The cat was more refractory to this effect than the rat and guinea pig. The rabbit did not appear to be susceptible to ammonium lung edema. (2) While a number of routes of administration of ammonium chloride were found effective for the production of acute pulmonary edema, the gavage route (0.09-0.15 g per 100 g of body weight) was most dependable for the guinea pig, and the intraperitoneal route (0.04 g per 100 g of body weight) for the rat. (3) The ammonium ion was found to be the agent responsible for the lung edema. Many ammonium salts produced this effect. Acidosis was ruled out as the primary factor, because acidosis produced in other ways did not result in lung edema. (4) The syndrome of ammonium intoxication consisted chiefly of dyspnea, muscle fasciculations and convulsions, terminating in an early acute pulmonary edema. (5) Gross changes at autopsy were limited to the thoracic cavity. They consisted of pulmonary edema and congestion with variable degrees of hemorrhage and moderate dilatation of the right ventricle, right atrium, and entering venae cavae. The microscopic appearance of the lungs was one of edema, congestion and hemorrhage.

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[9] v. Fazekas, G. I., *Deutsch. Ztschrft. f.d. ges. gericht. Med.*, 1934, **23**, 225.

[10] McNeil, H. L., and Levy, M. D., *J. Lab. and Clin. Med.*, 1917, **3**, 18.

[11] Keith, N., quoted by Sollmann, T., A Manual of Pharmacology, W. B. Saunders, Co., 7th edition, 1948, 776.

16932

Changes in Blood Chemistry in Rats Following Electrically-Induced Seizures.*

John R. Ward† and Lloyd S. Call. (Introduced by Horace W. Davenport.)

From the Department of Physiology, University of Utah College of Medicine, Salt Lake City.

A maximal seizure consists of violent neural and muscular activity lasting many seconds and might be expected to involve considerable changes in brain and blood chemistry. Klein and Olsen[1,2] found that 10 seconds of convulsive activity accelerates the glucose metabolism of brain and results in an approximately two-fold increase in brain lactate and a decrease in high-energy phosphate bonds. McLaughlin and Hurst[3] observed moderate decreases in plasma pH and alkali reserve and corresponding increases in blood lactate, and others[4-7] have found increases in plasma phosphate following idiopathic convulsions in humans. The post-convulsive depression and other sequelae of seizures may be the result of metabolic activity occurring during seizures. In order to determine the magnitude and duration of chemical changes occurring in a commonly used experimental animal, the work presented here was undertaken.

Methods. Adult male rats of the Sprague-Dawley strain were used. Maximal seizures were induced by passing a 150 mA current of 0.2 secs duration between Spiegel corneal electrodes.[8] Blood was obtained by heart puncture, and heparin was used as an anticoagulant. Plasma was separated anaerobically by the method of Davenport.[9] Its pH was determined by means of a standardized glass electrode, and its carbon dioxide by the method of Van Slyke and Neill.[10] Plasma lactate was determined by the method of Barker and Summerson,[11] and plasma inorganic phosphate was determined by the method of Fiske and SubbaRow.[12] Plasma sodium and potassium were estimated by means of a Perkin-Elmer flame photometer (Model 52A) with internal lithium standard.

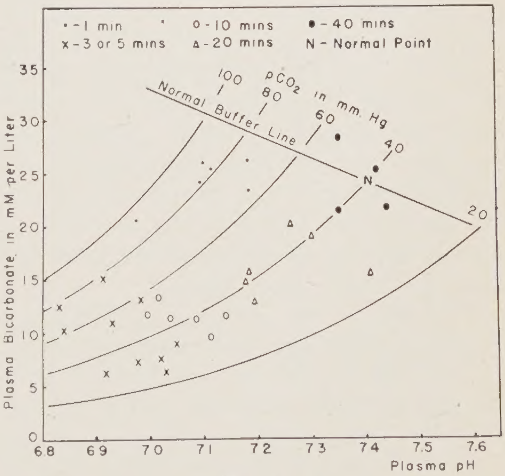

Fig. 1.

pH-bicarbonate diagram showing blood acid-base changes following maximal seizures in rats.

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* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

† Fellow of the University of Utah Research Fund.

[1] Klein, J. R., and Olsen, N. S., *J. Biol. Chem.*, 1947, **167**, 747.

[2] Olsen, N. S., and Klein, J. R., *Proc. A. Res. Nerv. and Ment. Dis.*, 1946, **26**, 118.

[3] McLaughlin, F. L., and Hurst, R. H., *Quart. J. Med.*, 1933, **2**, 419.

[4] Goldstein, H., and McFarland, R. A., *Am. J. Psych.*, 1939, **96**, 771.

[5] del Roncal, F. P., *J. Nerv. and Ment. Dis.*, 1941, **94**, 133.

[6] Weil, A., and Liebert, E., *Arch. Neur. and Psych.*, 1937, **37**, 584.

[7] Hirschfelder, A. D., and Haury, V. G., *Arch. Neur. and Psych.*, 1938, **40**, 66.

[8] Toman, J. E. P., Swinyard, E. A., and Goodman, L. S., *J. Neurophysiol.*, 1946, **9**, 231.

[9] Davenport, H. W., *Science*, 1947, **105**, 2717.

[10] Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

[11] Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, 1941, **138**, 535.

[12] Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.*, 1925, **66**, 375.

TABLE I.
Concentrations of Electrolytes in Rat Plasma Following Maximal Electroshock Convulsions.

Time after convulsions, min.	Lactate, mEq/l	Phosphate, mM/l	Sodium, mEq/l	Potassium, mEq/l
Control	3.1 ± 0.2* (12)	1.9 ± 0.1 (8)	142.5 ± 0.1 (12)	4.8 ± 0.2 (12)
1	20.8 ± 1.9 (6)	3.4 ± 0.3 (6)	155.1 ± 1.1 (6)	5.1 ± 0.1 (6)
5	20.3 ± 1.5 (6)	2.8 ± 0.3 (6)	151.1 ± 0.6 (6)	4.4 ± 0.1 (6)
10	—	—	147.2 ± 0.2 (6)	4.0 ± 0.2 (6)
20	11.1 ± 1.6 (6)	2.5 ± 0.1 (6)	147.3 ± 1.1 (6)	3.8 ± 0.2 (6)
40	3.9 ± 0.5 (6)	2.1 ± 0.1 (6)	140.6 ± 0.4 (5)	4.6 ± 0.1 (5)
60	3.7 ± 0.6 (6)	1.8 ± 0.1 (6)	—	—

* Standard error of the mean.

Results. The plasma pH and bicarbonate concentration, observed at intervals after seizures, are plotted on the pH-bicarbonate diagram shown in Fig. 1. The plasma lactate, phosphate, sodium and potassium concentrations are given in Table I.

One minute after a maximal seizure, respiratory acidosis was present, the pCO_2 averaging 80 mm Hg. There was also a severe metabolic acidosis. Total extra fixed-acids of the blood were greater than 20 mEq/l, and the sodium concentration had risen 12 mEq/l. At the end of 5 minutes the respiratory acidosis had disappeared; but the metabolic acidosis was still extreme, and all observed pH values were below 7.05. Thereafter the metabolic acidosis became progressively less severe until recovery was attained at 40 minutes. Simultaneously the plasma sodium concentration returned to normal, and the plasma potassium concentration fell slightly below normal.

Discussion. The metabolic changes produced by a maximal seizure can be explained by the vigorous muscular activity and cessation of respiration occurring during the convulsion. The acidosis is as severe as that observed in completely uncontrolled diabetes mellitus, and the rise in sodium concentration is equal to that produced by overdosage with desoxycorticosterone acetate.[13] It is likely that the metabolic changes, rather than purely electrical events, in the brain, account for most of the post-seizure depression whose time course parallels that of the chemical changes in the blood.[8] Extreme metabolic acidosis, produced by ammonium chloride, itself reduces the excitability of the brain as measured by an increase in electroshock threshold.[14] A rise in plasma sodium concentration is similarly associated with reduced cerebral excitability.[13] If similar changes occur in humans undergoing electroshock therapy of psychotic disorders, it would appear important to investigate the relative contributions of the changes in brain and blood chemistry and the electroshock itself to the salutary results of the treatment.

Summary. Severe respiratory and metabolic acidosis, accompanied by increases in plasma lactate, phosphate and sodium concentrations, occur in rats following maximal electrically-induced seizures. Recovery is complete in 40 minutes. It is suggested that the profound chemical changes occurring during the seizure may contribute to the post-ictal depression.

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[13] Woodbury, D. M., and Davenport, V. D., in press.

[14] Hendley, C. D., Davenport, H. W., and Toman, J. E. P., *Am. J. Physiol.*, 1948, **153**, 580.

16933

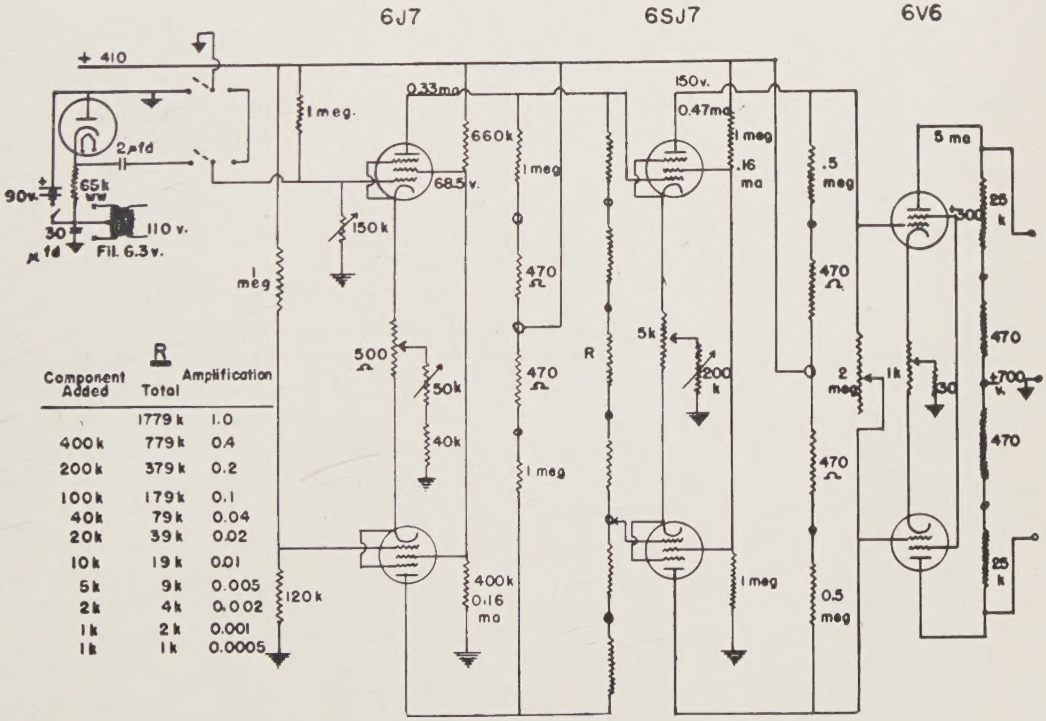
Application of the Transducer Tube to the Recording of the Peripheral Pulse.

Howard J. Curtis* and John L. Nickerson.

From the Department of Physiology, College of Physicians and Surgeons, Columbia University, New York.

The observation and recording of the form of the venous, radial or other peripheral pulses in man has some value in teaching the dynamics of the circulation as well as in aiding clinical diagnosis.[1] Conventionally the pulsation is picked up by a cup-like device pressed over the area involved. The impulses are transmitted by rubber tubing to a tambour or segment capsule for recording.

This paper presents the application of the Transducer Tube (RCA Developmental Tube No. C 798 B) to this problem. In this tube the plate is connected to a peg extending through a flexible diaphragm to the outside so that micromovements of the peg will produce changes in the plate current. A light weight button and lever are attached to the peg. The button is touched to the pulsating area and its movements are transformed to current changes, amplified and recorded by an ink writing crystograph (Fig. 1) or by a galvanometer recording on photographic paper. The natural frequency of the moving part is remarkably high and with careful mechanical design and a light weight button it can be as high as 100 cycles per second, a value which



Component Added	R Total	Amplification
	1779 k	1.0
400 k	779 k	0.4
200 k	379 k	0.2
100 k	179 k	0.1
40 k	79 k	0.04
20 k	39 k	0.02
10 k	19 k	0.01
5 k	9 k	0.005
2 k	4 k	0.002
1 k	2 k	0.001
1 k	1 k	0.0005

Fig. 1.

The transducer circuit with its amplifier of successive push-pull stages of 6J7, 6SJ7, and 6V6 to the direct writer is shown.

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* Now at the Department of Physiology, Vanderbilt University, Nashville, Tenn.

[1] Wiggers, C. J., Physiology in Health and Disease.

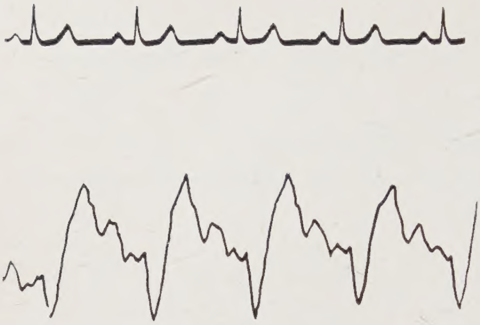

FIG. 2.
Illustrates the simultaneous record of the jugular pulse and the electrocardiogram.

compares favorably with the best devices in the literature. In Fig. 2 is shown a record of the venous pulse with a simultaneous recording of the electrocardiogram.

In presenting this application of the transducer tube it is desired to point out that this is only one of its many possible uses and that it should have rather wide usefulness in biology and medicine. The tube itself is about the size of the end of a pencil and can thus be used in small places if necessary. Furthermore, since the plate current in the transducer tube depends upon the displacement of the plate it is possible with a slight change in the circuit of Fig. 1 to measure displacements rather than changes in displacement. The sensitivity is high, and it has been our experience that it is easier to obtain a given sensitivity with a transducer than with a resistance wire strain gage.

Summary. This paper presents the application of the transducer tube to the detection and recording of movements of the type associated with the peripheral venous pulses. An adequate amplifier circuit is given and the usefulness of the device in other applications is suggested.

16934

The Fate of C^{14}-Labeled Palmitic Acid Administered Intravenously as a Tripalmitin Emulsion.*†

S. R. LERNER, I. L. CHAIKOFF, C. ENTENMAN, AND W. G. DAUBEN.

From the Division of Physiology of the Medical School and the Department of Chemistry, University of California, Berkeley.

The need for providing sufficient calories in small volumes for parenteral feeding has focussed attention upon methods for the intravenous administration of fat in a highly emulsified form. Now that several investigators have shown that the intravenous administration of fat can be achieved with some degree of safety,[1-3] it has become of importance to determine whether fat so administered pursues a normal metabolic path. Several attempts have been made to study the utilization of parenterally administered fats but the results obtained until recently were not conclusive. Thus Dunham and Brunschwig[4] failed to observe protein-sparing effects in 9 of 11 dogs when a highly emulsified fat was injected intravenously for periods as long as one month. Their emulsions, however, were quite toxic. McKibbin *et al.*,[1] on the other hand, have concluded that intravenously administered emulsions of fat are

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[*] Aided by grants from the American Cancer Society (recommended by the Committee on Growth), and the Cutter Laboratories, Berkeley.

[†] A preliminary report of the results of the present investigation has appeared in *Science.*

[1] McKibbin, J. M., Pope, A., Thayer, S., Ferry, R. M., Jr., and Stare, F. J., *J. Lab. Clin. Med.*, 1945, **30**, 488.

[2] Meng, H. C., and Freeman, S., *J. Lab. Clin. Med.*, 1948, **33**, 689.

[3] Shafiroff, B. G. P., Baron, H., and Roth, E., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 387.

[4] Dunham, L. J., and Brunschwig, A., *Arch. Surg.*, 1944, **48**, 395.

TABLE I.
Recovery of C^{14} Activity in the Fatty Acids Isolated from Organs and Tissues 24 Hours After the Injection of C^{14}-Palmitic Acid as Tripalmitin.

Tissues	Rat 1 (188 g)	Rat 2 (152 g)	Rat 3 (161 g)	Rat 4 (159 g)
	% of the injected C^{14} recovered			
Liver	2.64	5.68	3.84	3.31
Lungs	0.58	2.58	0.24	0.14
Spleen	0.51	1.01	0.10	0.26
Kidney	0.42	0.33	—	—
Small Intestine*	1.33	1.54	0.95	0.79
Large Intestine*	—	—	0.13†	0.13†
Brain	—	—	0.05	0.05
Depot:				
Perirenal	0.30	0.26	—	—
Genital	0.31	0.34	—	—
Omental	0.21	0.09	—	—
Mesenteric	0.74	0.39	—	—
Skin‡	6.6	3.2	—	—
Carcass§	5.6	6.6	15.9	13.8
Feces	0.76‖	1.13‖	0.36	0.32
Total	19.8	22.1	21.6	18.8

* Washed free of intestinal contents as described in text.
† The large intestines of rats 3 and 4 were pooled; 0.13 is half of the total C^{14} recovered.
‡ Includes hair and subcutaneous fat
§ Includes all organs and tissues not specified above.
‖ The value includes the activity of the feces plus that of the large intestine and its contents.

utilized, for not only did they find weight improvement and nitrogen retention in 2 dogs, but they were also unable to find any considerable portion of the infused fat stored in an unmodified form. A gain in body weight in dogs that received fat emulsions intravenously was also noted by Meng and Freeman,[2] but they point out that such results furnish no direct proof of fat utilization.

In order to study the path of utilization of fat introduced by the intravenous route, palmitic acid labeled at its 6th carbon with C^{14} was used in the present investigation. It is shown here that palmitic acid, when injected directly into the blood stream in the form of highly emulsified tripalmitin, is oxidized at a rapid rate, converted to phospholipid, and deposited in the fat depots.

Experimental. Preparation of emulsion. Palmitic acid containing C^{14} in the sixth carbon atom was synthesized, as described in an earlier communication,[5] and then esterified with glycerol by a modification of the method of Feuge *et al.*[6] The labeled tripalmitin was melted, together with half its weight of glycerol monostearate, and enough 5% glucose solution was added to yield an emulsion containing 4% fat. A propeller attached to a high speed motor was then inserted and the mixture was agitated violently for 5 minutes at a temperature maintained near the boiling point. The resulting emulsion was irradiated with supersonic energy (Ultra-sonorator, Model SL520) for 10 minutes at 700,000 cycles per second. The usual volume so treated was 3-5 ml. The particles obtained were almost all less than 1 μ in diameter.

Treatment of animals and collection of expired $C^{14}O_2$. Rats of the Long-Evans strain, weighing from 152 to 188 g, were used. After being fasted for 24 hours, they were anesthetized lightly with ether. One or 1.5 ml of the emulsion containing 20-25 mg of labeled tripalmitin (from 4 to 7 × 10^4 counts per minute per mg tripalmitin) were then slowly injected into the foot vein of each rat. The injection lasted about one minute. The rats were then placed in large pyrex desiccators arranged so that urine and feces could be collected and CO_2-free air could be drawn

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[5] Dauben, W. G., *J. Am. Chem. Soc.*, 1948, **70**, 1376.

[6] Feuge, R. O., Kraemer, E. A., and Bailey, A. E., *Oil and Soap*, 1945, **22**, 202.

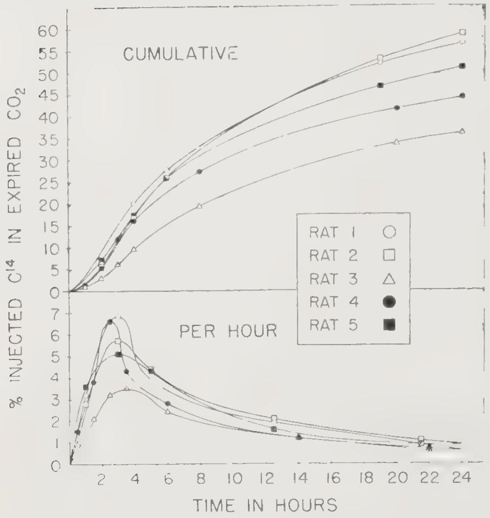

Fig. 1.

Rate of conversion of intravenously injected C^{14}-labeled tripalmitin to CO_2.

through. Water, but not food, was provided. The air leaving the desiccator was dispersed, by a sintered glass disc, into a tower containing a sodium hydroxide solution. The sodium hydroxide was changed at intervals so that collection times were 0-2, 2-4, 4-6, 6-19, and 19-24 hours for three rats, and 0-1, 1-2, 2-3, 3-4, 4-8, 8-20, and 20-24 hours for 2 rats.

The expired CO_2 collected as the carbonate was precipitated as $BaCO_3$ and prepared for counting as described previously.[7]

Treatment of tissues. At the end of 24 hours, the rats were deeply anesthetized with ether. The organs and tissues indicated in Table I were removed and covered with alcohol. The small and large intestine were washed free of their contents with cold ethyl alcohol and these contents added to the collected feces. All remaining tissues were labeled carcass. Saturated potassium hydroxide solution (1 cc per g tissue) was added to each tissue sample and the fats were saponified. After saponification, the solutions were made acid to methyl red with sulfuric acid and the fatty acids extracted with ether. Aliquots of the ether extracts were mounted on lens-paper-covered aluminum discs for determination of their radioactivity, as described elsewhere.[7]

The time required to yield 1024 counts was determined several times for each sample. A Geiger-Müller tube with a mica window of 1.7 mg per sq. cm was used. All counting rates were at least several times that of background.

Results. The rapidity with which the intravenously injected tripalmitin is oxidized by fasted rats is shown in Fig. 1. Significant amounts of C^{14} appeared in the expired CO_2 as early as one hour, and by the time 24 hours had elapsed, from 36 to 59% of the sixth carbon atoms of the palmitic acid molecules were recovered as $C^{14}O_2$. The rate of elimination of $C^{14}O_2$ was not uniform throughout the period studied. It was most rapid between the second and fourth hours—from 19 to 25% of the total 24-hour elimination was expired during these 2 hours.

The recovery, at the end of 24 hours, of the injected C^{14} among the fatty acids isolated from various tissues is shown in Table I. Significant amounts, namely, 6-8%, were found in the areas designated as fatty depots and of these, the largest amount was present in the subcutaneous fat. If these values are considered in conjunction with the amount of C^{14} undoubtedly present in intermuscular fat of the carcass, there can be no doubt that fatty depots constitute a most important metabolic pathway for *intravenously* administered fat.

The only other tissue that contained appreciable amounts of the injected C^{14} was the liver. One day after the administration of the labeled palmitic acid, approximately 3-6% of the C^{14} was recovered in the fatty acid fraction of this tissue. Negligible amounts of C^{14} were found in the brain, spleen, lungs, and kidneys.

That the intravenously administered emulsified tripalmitin is available for phospholipid formation is shown in Table II. In rats 3 and 4, the major portion (70-80%) of the C^{14}-labeled fatty acids in the liver and small and large intestine had been incorporated into phospholipids. This was also true for the small intestine of rats 1 and 2, but in the case of

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[7] Entenman, C., Lerner, S. R., Chaikoff, I. L., and Dauben, W. G., Proc. Soc. Exp. Biol. and Med., in press.

TABLE II.
Incorporation of Labeled Fatty Acids into Phospholipid and Nonphospholipid Fractions of Tissues 24 Hours After the Injection of C^{14}-Palmitic Acid as Tripalmitin.

Tissue	Lipid fractions	Rat 1	Rat 2	Rat 3	Rat 4
		% of each tissue's lipid C^{14} found in respective fraction			
Liver	Phospholipid fatty acids	34	50	72	72
	Nonphospholipid "	66	50	28	28
Small Intestine	Phospholipid "	64	71	74	78
	Nonphospholipid "	36	29	26	22
Large Intestine	Phospholipid "	—	—	69*	69*
	Nonphospholipid "	—	—	31*	31*

* The large intestines of rats 3 and 4 were pooled and the value shown is half of the total.

their livers, 34 and 50%, respectively, of their fatty acids were in the phospholipid fraction.

Comment. The manner in which long-chain fatty acids are utilized when administered by routes other than the gastrointestinal tract has aroused considerable interest in recent years. The use of radioactive carbon provided, for the first time, a direct method for determining the rate at which an animal can convert parenterally administered, long-chain fatty acids to CO_2. In the present investigation, a 16-carbon fatty acid containing C^{14} in the sixth carbon atom was used and injected in the form of its triglyceride. It is shown here that as much as 59% of the sixth carbon atoms are converted to CO_2 in 24 hours. This observation, in addition to the demonstration of storage of significant amounts of the labeled fatty acids in adipose tissues and their recovery in the phospholipids isolated from liver and intestine, can leave no further doubt that palmitic acid is utilized when used for intravenous feeding.

Since the completion of the present study, Geyer *et al.* have reported on the oxidation of intravenously administered trilaurin.[8] They injected a fat emulsion containing trilaurin in which the carboxyl group of the 12-carbon fatty acid was labeled with C^{14}, and found that in 4.5 hours, 71% of the C^{14} injected into rats was exhaled as CO_2. In this interval we found that only 11-23% of the sixth carbon atoms of palmitic acid, which had been administered intravenously as a tripalmitin emulsion, are exhaled as CO_2. The more rapid oxidation rate of trilaurin is most likely due to the fact that the 12-carbon fatty acid is not deposited in adipose tissue to a measurable extent.

Summary. 1. In order to test the extent of utilization of fat emulsions in parenteral feeding, palmitic acid labeled with C^{14} at its sixth carbon was injected intravenously, in the form of the triglyceride, into fasted rats.

2. From 36 to 59% of the administered C^{14} was expired as CO_2 in 24 hours.

3. Considerable amounts of the administered fatty acids were stored in adipose tissues throughout the body.

4. The availability of intravenously administered tripalmitin for metabolic purposes is further shown by the finding that as much as 78% of the radioactive fatty acids recovered from the liver and small intestine had been incorporated into phospholipids.

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[8] Geyer, R. P., Chipman, J., and Stare, F. J., *J. Biol. Chem.*, 1948, **176**, 1469.

16935

A Fat Emulsion for Intravenous Feeding.*

S. R. LERNER, I. L. CHAIKOFF, AND C. ENTENMAN.

From the Division of Physiology, University of California Medical School, Berkeley, Calif.

Attempts to utilize fat for intravenous feeding, because of its high caloric content, have led to the development of many types of fat emulsions.[1-9] The special problems encountered in such usage involve the dispersion of the fat into suitable particle sizes (0.5 to 1 μ) and the stabilization of that dispersion. The former has been achieved by high pressure homogenization of such fats as coconut, olive, corn, and butter oils. Egg or soya phosphatides have, in most cases, been used as stabilizers.

McKibbin *et al.* devised an emulsion of coconut oil which was stabilized with soya phosphatides.[6] It was well tolerated when injected slowly, but foreign body responses were observed following chronic injections. The investigators reported, however, that impurities in the soya phosphatides caused the foreign body reactions and that simple purification freed the emulsion of toxicity.[10] These findings have been confirmed by Ng in this laboratory.[11] He found that daily intravenous injections of a commercially prepared soya phosphatide, for 15-25 days, produced a mild hemolysis and a lipid-storage reaction in the reticulo-endothelial system. Use of the fraction of this commercial preparation which was soluble in 95% ethyl alcohol at 60° did not prevent the untoward reactions.

Meng and Freeman devised an emulsion of butter oil stabilized by the presence of a polyalcohol derivative and soya phosphatides, in an oil phase, and sodium cholate in an aqueous phase.[7] This emulsion was tolerated during its intravenous administration and, as shown by postmortem examination, resulted in only minimal changes in lungs and kidneys of dogs that had received daily injections for several weeks.

Shafiroff and Frank prepared an emulsion of coconut oil in a menstruum composed of 6% gelatin, 5% protein hydrolysate, and 5% glucose.[8,9] Apparently it was well tolerated for they observed no evidence of toxicity.

In the present investigation, a fat emulsion not hitherto used for intravenous feeding is described. This consisted of an olive oil dispersion stabilized by means of glycerol monostearate. The daily intravenous injection of this emulsion for as long as 2 to 4.5 weeks produced only minimal reactions in dogs as judged by microscopic examination of their tissues.

Experimental. Preparation of emulsion. Two parts of olive oil and one part glycerol monostearate[†] were heated, with stirring, until

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* Aided by a grant from the Cutter Laboratories, Berkeley.

[1] Nomura, T., *Tohoku J. Exp. Med.*, 1929, **12**, 247.

[2] Holt, L. E., Jr., Tidwell, H. C., and Scott, T. F., *J. Pediatrics*, 1935, **6**, 151.

[3] Clark, D. E., and Brunschwig, A., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 329.

[4] Myers, R. J., and Blumberg, H., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 79.

[5] Dunham, L. J., and Brunschwig, A., *Arch. Surg.*, 1944, **48**, 395.

[6] McKibbin, J. M., Pope, A., Thayer, S., Ferry, R. M., Jr., and Stare, F. J., *J. Lab. Clin. Med.*, 1945, **30**, 488.

[7] Meng, H. C., and Freeman, S., *J. Lab. Clin. Med.*, 1948, **33**, 689.

[8] Shafiroff, B. G. P., and Frank, C., *Science*, 1947, **106**, 474.

[9] Shafiroff, B. G. P., Baron, H., and Roth, E., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 387.

[10] Geyer, R. D., Mann, G. V., Young, J., Kinney, T. D., and Stare, F. J., *L. Lab. Clin. Med.*, 1948, **33**, 163.

[11] Ng, E., unpublished observations.

[†] A commercial preparation manufactured by Glyco Products Co., Inc., Brooklyn, New York, and designated Aldo 28, was used. This product is glycerol monostearate modified by the inclusion of a small amount of a sodium soap. Emulsions prepared with unmodified glycerol monostearate gelled a few hours after autoclaving.

a clear solution was obtained. This melt was then added to a sufficient volume of 5% glucose to yield a final concentration of 10% fat, and the mixture was stirred in a Waring blender for 3-5 minutes. Three gallons of this preliminary emulsion were passed through a dairy homogenizer 5 times at 2500 lb. per sq. inch. The diameters of the fat particles of the final emulsion were determined by visual measurement with an ocular micrometer and, as a rule, found to be one micron or less. Particles as large as 2 μ were only occasionally observed. The pH of this emulsion was 7.0. Such emulsions contained about 10% fatty acids of which 2/3 were derived from olive oil and 1/3 from glycerol monostearate. Bottling in cotton-stoppered 500 cc Erlenmeyer flasks, autoclaving for 30 minutes at 5 lb., and cooling in an ice bath produced a sterile emulsion that creamed only slightly after 2 months. The emulsion was stored at room temperature.

Treatment of dogs. For several days before injection and throughout the injection period, the dogs were fed once daily a mixture composed of 40 g of lean horse meat per kg body weight, 20 g of sucrose, 20 g of bone ash, one cc of fish oil,[‡] and 10 g of yeast. Before the start of the injection, samples of blood were withdrawn for determination of red cell counts and hemoglobin concentration. Liver function also was measured by means of the Rose Bengal test as described by Hough and Freeman.[12]

The dogs were injected with 200 cc of the fat emulsion, either daily or 6 days per week. The dogs received, in all, 16 to 31 intravenous injections. A few days before the last injection, evidence of toxicity was sought by determination of red cell counts, hemoglobin concentrations of blood, and of liver function.

Methods. The method used for the determination of fatty acid contents of plasma and

TABLE I.

Effect of Intravenous Injections of a Fat Emulsion on Dogs.
(Each dog received 200 cc of 10% fat emulsion at each injection.)

		Wt		Hemoglobin		Red blood cell count		Rose Bengal clearance		
Dog	No. of inj.	Before inj. started, kg	At end of inj. period, kg	Before inj. started, g per 100 ml	On indicated day of inj. period, g per 100 ml	Before inj. started, ×10⁶	On indicated day of inj. period, ×10⁶	Before inj. started	On indicated day of inj. period	Liver fatty acid, % wet wt
1	13	12.7	12.5	16.0	13.0 (13)[*]	8.1	7.1 (13)[*]	136[†]	114 (13)[*]	3.1
2	24	8.7	8.5	13.2	10.8 (20)	7.1	4.7 (20)	130	82 (20)	2.9
3	16	15.1	15.4	15.6	12.5 (15)	7.6	6.6 (15)	114	110 (15)	3.2
4	16	10.2	10.3	14.0	12.6 (15)	7.7	6.4 (15)	114	82 (15)	4.1
5	31	13.2	12.5	16.0	9.5 (31)	8.7	5.2 (31)	104	108 (31)	—

[*] Numbers in parentheses indicate the day of the injection when test made.
[†] Values lower than 100 indicate impaired excretion.[12]

[‡] Each cubic centimeter of the fish oil (sardilene) contained not less than 100 A.O.A.C. chick units of vitamin D and 600 U.S.P. units of vitamin A.

[12] Hough, V. H., and Freeman, S., *Am. J. Physiol.*, 1942, **138**, 184.

liver has been recorded elsewhere.[13,17] Red cell counts and hemoglobin concentrations of blood were determined as described by Gradwohl.[14]

Results. The 200 cc of the emulsion which each dog received daily by intravenous drip contained 13.3 g of olive oil and 6.7 g of glycerol monostearate, or a total of 20 g of fatty acids. It was administered at a rate of about 1 cc per minute per kg of body weight. No evidence of distress other than a slight restlessness was observed during the injections which occupied, as a rule, from 20 to 30 minutes. Immediately after completion of the injection, the dogs were fed their daily ration which was readily consumed.

Five dogs were used in this investigation (Table I). They received 16 to 31 daily injections. Red cell counts, hemoglobin concentrations of the blood, and liver function were measured on the day before the injections were begun and at designated days near the end of the injection period.

Before the injections were begun, the hemoglobin content of the blood of the 5 dogs ranged from 13.2 to 16.0 g per 100 cc. These values are well within the range reported by other investigators.[6,7,15] The concentration of hemoglobin in all 5 dogs had decreased by the time the injections had continued for 13 to 31 days. This is in keeping with the observed fall of the red cell counts. Similar hematological findings were reported by McKibbin *et al.*[6] and by Meng and Freeman[7] in their dogs that received injections of fat emulsions. Despite the slight anemia a weight loss was not observed in 4 dogs that received as many as 31 daily infusions. Dogs 1, 3, and 5 showed no evidence of impaired liver function as judged by the rate at which injected Rose Bengal was lost from the blood stream.

TABLE II.
Rate of Disappearance of Injected Fat from the Blood Stream of the Dog.
(All lipid values expressed as mg per 100 cc plasma).

Dog 1*			Dog 2†			Dog 3†		Dog 4†		Dog 5‡			
Interval after inj., min.	Total fatty acids	Total cholesterol	Interval after inj., min.	Total fatty acids	Total cholesterol	Interval after inj., min.	Total fatty acids	Interval after inj., min.	Total fatty acids	Interval after inj., min.	Total fatty acids	Total cholesterol	Phospholipid
—12§	356	166	—6§	338	167	—1§	317	—2§	194	—21§	456	213	364
9	1510	169	2	1310	134	5	1170	4	1940	9	1110	163	282
94	665	172	38	819	159	44	710	23	2000	49	766	198	321
246	412	167	72	582	142	99	410	69	1150	136	526	199	328
535	405	172	132	465	155	160	317	152	402	202	431	196	328
			189	472	157			217	293				

* This dog received a total of 13 injections and the data shown above are for the eighth injection.
† The measurements recorded above were made on the day of the last injection.
‡ This dog received a total of 31 injections and the data shown above are for the 26th injection.
§ Minus values indicate the time in minutes before the injections were started.

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[13] Chaikoff, I. L., and Kaplan, A., *J. Biol. Chem.*, 1937, **119**, 423.

[14] Gradwohl, R. B. H., Clinical Laboratory Methods and Diagnosis, 3rd ed., 1943.

[15] Allison, J. B., *Trans. N. Y. Acad. Sci.*, Ser. II, 1946, **8**, 260.

[16] Entenman, C., Chaikoff, I. L., and Zilversmit, D. B., *J. Biol. Chem.*, 1946, **166**, 15.

[17] Chaikoff, I. L., and Kaplan, A., *J. Biol. Chem.*, 1934, **106**, 267.

The excretion of the dye by dogs 2 and 4 was 82% of normal.

The rate of disappearance of the injected fat from the blood stream was measured for each dog and the results are shown in Table II. The first examination of plasma fatty acids was made from 2 to 9 minutes after the completion of the injection, and at these times fatty acid concentrations were between 1110 and 1940 mg %. Utilization of the injected fat, as judged by its disappearance from the blood stream, was rapid in all 5 dogs, and by the time 4 hours had elapsed, the concentration of plasma fatty acids approached the preinjection levels.

The dogs were sacrificed by an overdose of nembutal 24 hours after the last injection. All organs were examined grossly and lungs, liver, kidney, spleen, small intestine, and heart studied microscopically. The lungs of all 5 dogs were free of lipoid granulomatous lesions and the lungs of only 2 dogs showed a few inflammatory foci. The reticulo-endothelial elements of the spleens and livers contained moderate amounts of blood pigments, a finding that indicates that some hemolysis had occurred. In dog 4, lipid-like material was found in the reticulo-endothelial cells of the liver and in the capillary loops of occasional glomerular tufts. Dog 2 had a few shrunken and scarred glomeruli adherent to the capsule. Protein containing fluid was found in the glomerular and tubular spaces. Dog 5 was pregnant and 2 of its 6 fetuses were dead. The fatty acid contents of the livers of dogs 1-4 were normal (Table I). The liver of dog 5, as judged by microscopic examination, was free of abnormal amounts of fat.

Comment. It is shown here that by the use of glycerol monostearate as stabilizer, an olive oil emulsion in which the fat remains dispersed for several months can be easily prepared. This emulsion is well tolerated by dogs when injected intravenously at rates of 1 cc per minute per kg, and is rapidly removed from the blood stream. Although dogs that had received as many as 31 injections were not free of tissue responses, the reactions observed were quite mild in character.

In confirmation of earlier findings,[1,2,4,7] it was observed here that intravenously administered fat rapidly disappears from the blood stream. The rate at which this occurs suggests that the administered fat is taken up by the liver for it has been shown that lipids disappear slowly, if at all, from the plasma of hepatectomized dogs.[16]

Summary. 1. The preparation of a stable olive oil emulsion in which glycerol monostearate was employed as the emulsifying agent is described. 2. Observations on its use for intravenous feeding in dogs are recorded.

16936

Excretion of 17-Ketosteroids by Patients Suffering from Poliomyelitis.*

Saul L. Cohen.

From the Department of Physiological Chemistry and the M. H. Hoffman Endocrinological Research Laboratory, University of Minnesota Medical School, Minneapolis, Minn.

It has long been recognized that the incidence of poliomyelitis is greater for children than for adults (Fanconi[1]). Since the excretion of 17-ketosteroids increases rapidly during adolescence it was thought possible that a low androgen excretion may be directly or indirectly associated with an increased incidence to poliomyelitis. The excretion of 17-ketosteroids by poliomyelitis patients was, therefore, investigated.

The urine specimens from a total of 39

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* Aided by a grant from the National Foundation for Infantile Paralysis.

[1] Fanconi, G., *Die Poliomyelitis und Ihre Grenzgehiete,* Benno Schwabe and Co., Basel, Switzerland.

TABLE I.
Excretion of 17-Ketosteroids by Patients Suffering from Poliomyelitis.

No. of patients	Sex	Age range	Type of poliomyelitis	Mg 17-Ks. excreted/24 hr range	avg
10	Male	5-13	Bulbar (4)	0.2-1.8	1.0
			Spinal (4)	0.8-4.8	2.5
			Bulbar-Spinal (2)	0.9-2.0	1.5
1	Female	7	Spinal	2.3	
15	Male	14-39	Bulbar (3)	11.4-36.0	19.0
			Spinal (8)	3.4-27.1	10.3
			Bulbar-Spinal (4)	14.9-22.1	19.0
13	Female	14-35	Bulbar (3)	3.1-7.2	5.4
			Spinal (8)	2.0-21.1	8.0
			Bulbar-Spinal (2)	8.6-14.6	11.6

patients entered at the University of Minnesota Hospital during the 1946 epidemic were studied. Of these, 11 were under age 13, 11 were females over age 14, and 15 were males over age 14. In some cases urine specimens were collected from the same patient on more than one occasion and these were subjected to separate analyses. All specimens had been stored in a frozen condition since the time of collection. The 17-ketosteroid fractions were prepared from 200-400 cc aliquots of 24-hour specimens by a modified Callow technic (Callow, *et al.*[2]) and were colorimetrically assayed by the technique of Holtorff and Koch[3]. The results are shown in Table I.

The values for the 17-ketosteroid excretion of the patients suffering from poliomyelitis for the most part fall within the normal ranges reported by others (cf. Talbot and Butler[4]) and as found in our own laboratory. These data thus tend to indicate that there is no relationship between the condition of poliomyelitis and the excretion levels of 17-ketosteroids. No significant differences were observed for the 17-ketosteroid excretion of patients suffering from different types of poliomyelitis.

Summary. The 17-ketosteroids excreted into the urine by patients suffering from different types of poliomyelitis showed no significant variations from normal excretion levels.

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[2] Callow, N. H., Callow, R. K., and Emmens, C. W., *Biochem. J.*, 1938, **32**, 1312.

[3] Holtorff, A. F., and Koch, F. C., *J. Biol. Chem.*, 1940, **135**, 377.

[4] Talbot, H. B., and Butler, A. M., *J. Clin. Endocrinology*, 1942, **2**, 724.

16937

Growth Promoting Activity of Vitamin B_{12} in Rats Receiving Thyroid Substance.

GLADYS A. EMERSON. (With the technical assistance of Marilyn A. Bingemann.)
From the Merck Institute for Therapeutic Research, Rahway, N. J.

The retarded growth observed in young rats fed toxic doses of thyroid powder is counteracted by liver.[1,2] Extracted Liver Residues (Wilson) had "anti-thyrotoxic" activity when fed at a level of 10% in a diet containing 22% casein.[3] Reticulogen also was effective in reversing the growth retarding action of thyroid substance in chicks fed a diet devoid of animal protein.[4,5]

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[1] Ershoff, B. H., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 500.

[2] Betheil, J. S., Wiebelhaus, V. D., and Lardy, H. A., *J. Nutrition*, 1947, **34**, 431.

[3] Ershoff, B. H., *Arch. Biochem.*, 1947, **15**, 365.

TABLE I.
Effect of Vitamin B_{12} on Growth of Male Rats Receiving Thyroid Powder.

Groups of 10 male rats each	Supplement	Gain in wt 15 days, g	Avg daily food intake 15 days, g
H_4 (Casein)	None	65	16
282 (Soybean meal)	,,	49	17
286 (Soybean meal + 0.25% thyroid powder)	,,	28	17
,, ,, ,, ,, ,, ,,	5.8 mg cobalt nitrate	27	—
,, ,, ,, ,, ,, ,,	0.5 γ B_{12} p.o.*	79	17
,, ,, ,, ,, ,, ,,	,, ,, ,, s.c.†	74	—
,, ,, ,, ,, ,, ,,	0.25 ,, ,, p.o.	72	—
,, ,, ,, ,, ,, ,,	,, ,, ,, s.c.	73	—
,, ,, ,, ,, ,, ,,	0.125 ,, ,, p.o.	65	—
,, ,, ,, ,, ,, ,,	,, ,, ,, s.c.	71	—
,, ,, ,, ,, ,, ,,	0.0625 ,, ,, p.o.	51	—
,, ,, ,, ,, ,, ,,	,, ,, ,, s.c.	50	—

* *Per os.*
† Subcutaneously.

Vitamin B_{12}, a crystalline compound which has been isolated from both liver[6] and fermentation sources,[7] neutralizes the growth inhibiting effect observed in immature rats following the feeding of a diet containing soybean meal and thyroid powder. The basal diet (282) employed in these studies had the following composition: soybean meal, 60; salts, 4; dextrose, 24; crisco, 10; cod liver oil, 2 g/100 g and micronutrients: thiamine, 1; riboflavin, 2; pyridoxine, 1; Ca pantothenate, 10; nicotinamide, 10; inositol, 5; p-aminobenzoic acid, 30; choline, 100; biotin, 0.05; pteroylglutamic acid, 0.20; α-tocopherol, 14.2; 2 Me 1:4 naphthoquinone, 14.2 mg/100 g. Desiccated thyroid U.S.P. (Armour) replaced dextrose at a level of 0.25% in diet 286. A purified ration (H_4),* containing 24% Labco casein, served as a standard for comparison.

Male rats, averaging 40 g in weight, were placed at weaning on each of the 3 dietary regimens. After 28 days on experiment the weight increments in g for the several groups were as follows: Diet H_4 (24% casein), 130; diet 282 (60% soybean meal), 120; and diet 286 (60% soybean meal and 0.25% thyroid powder), 80. Following this depletion period the rats receiving diet 286 were segregated into groups as indicated in Table I.

The animals receiving 0.125 γ vitamin B_{12} daily in addition to diet 286 grew over a 15-day test period at a rate which was more than twice that of the controls. Their weight gain, over this period, approximated that of animals receiving the diet containing casein and exceeded that made by the rats fed the ration containing soybean meal without thyroid substance. This level appeared to approach the optimal as indicated by the fact that the feeding of higher levels of B_{12} resulted in only slightly increased weight increments. Vitamin B_{12} appeared to be utilized equally well when administered by either the oral or subcutaneous routes. Rats given cobalt nitrate by the oral route made

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[4] Robblee, A. R., Nichol, C. A., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 400.

[5] Nichol, C. A., Robblee, A. R., Cravens, W. W., and Elvehjem, C. A., *J. Biol. Chem.*, 1947, **170**, 419.

[6] Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K., *Science*, 1948, **107**, 396.

[7] Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K., *Science*, 1948, **108**, 634.

* Composition of Diet H_4	g/100 g
Casein (Labco)	24
Dextrose	60
Salts (U.S.P. No. 1)	4
Hydrogenated veg. oil	10
Cod liver oil	2

Micronutrients, mg/100 g: thiamine, 1; riboflavin, 2; pyridoxine, 1; Ca pantothenate, 10; nicotinamide, 10; inositol, 5, and choline chloride, 100.

weight gains comparable with those of the controls indicating that cobalt could not be employed under these conditions in the biosynthesis of vitamin B_{12}.

It is of interest to note that the food consumption over the 15-day test period for the rats receiving 0.5 γ of vitamin B_{12} daily and those fed the unsupplemented diet but without thyroid powder were the same. The red and white blood cell counts in both the control group and in the groups receiving vitamin B_{12} fell within the normal limits.

Summary. Vitamin B_{12} counteracts the growth retarding effect of thyroid powder when fed in conjunction with a diet devoid of animal protein. The vitamin is equally effective when administered by either the oral or the subcutaneous route.

16938

Entrance of Intravenously Injected Sucrose into the Cerebrospinal Fluid.*

ROGER S. HUBBARD AND JOHN ZOLL.

From the Department of Pharmacology, the Medical School, University of Buffalo, and the Millard Fillmore Hospital, Buffalo, N. Y.

Sucrose injected intravenously into human subjects can be recovered almost quantitatively from the urine.[1] It has been used as an osmotic diuretic drug[2] although its toxic actions greatly limit its usefulness.[3] The excretion of the sugar by the kidneys appears to result from quantitative filtration through the glomeruli, and, since the compound is very little affected by tubular reabsorption, its rate of excretion has been used to determine glomerular filtration[4] although sucrose is not apparently as satisfactory as inulin for this purpose.

Besides its action as a diuretic, it has been used in the study of body fluids. It appears not to penetrate cells readily[1] and to be contained almost solely in the extra-cellular fluid. Lavietes and his coworkers[5] have suggested that it be used to determine total extracellular fluid when large accumulations of abnormal fluid are absent. On the other hand sucrose appears to penetrate poorly into the spinal canal[6] and has been injected intravenously to reduce intracranial pressure by osmotic action.[7]

Hubbard and Anderson[8] developed a specific method for determining sucrose, injected large amounts of the sugar into human subjects who showed unusual accumulations of extra-cellular fluid, and compared the concentrations of sucrose in blood plasma and extracellular fluid samples withdrawn after various time intervals had elapsed. They found that sucrose diffused readily into extracellular

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* Our thanks are due to Dr. William Beswick for the opportunity for studying patients receiving treatment for increased intracranial pressure and to Dr. Wilbert H. Spencer for assistance with the bacteriological part of the study.

[1] Keith, N. M., and Powers, M. H., *Am. J. Physiol.*, 1937, **120**, 203.

[2] Goodman, L., and Gilman, A., The Pharmacologic Basis of Therapeutics, New York, 1941, p. 632.

[3] Helmholtz, H. F., *J. Pediat.*, 1933, **3**, 144; Anderson, W. A. D., and Bethea, W. R., *J.A.M.A.*, 1940, **114**, 983; Lindberg, H. S., Wald, M. H., and Barker, M. H., *Arch. Int. Med.*, 1939, **63**, 907.

[4] Joliffe, N., Shannon, J. A., and Smith, H. W., *Am. J. Physiol.*, 1932, **100**, 301; Shannon, J. A., and Smith, H. W., *J. Clin. Invest.*, 1935, **14**, 393.

[5] Lavietes, P. H., Bourdillon, J., and Klinghoffer, K. A., *J. Clin. Invest.*, 1936, **15**, 261.

[6] Gregerson, M. I., and Wright, L., *Am. J. Physiol.*, 1935, **112**, 97.

[7] Bulloch, M. T., Gregerson, M. I., and Kiney, R., *Am. J. Physiol.*, 1935, **112**, 82; Masserman, J. H., *Bull. Johns Hopkins Hosp.*, 1935, **57**, 12; Jackson, H., Dickerson, D., and Gunther, A., *Am. Surg.*, 1937, **106**, 161.

[8] Hubbard, R. S., and Anderson, R. K., *Am. J. Physiol.*, 1942, **137**, 722.

TABLE I.
Comparison of Sucrose in Serum and Spinal Fluid.

No.	Pathological condition	Time after sucrose inj., min.	Sucrose in serum, mg per 100 cc	Sucrose in spinal fluid, mg per 100 cc
1.	Brain tumor post operative	15	134	trace*
2.	Chronic subdural hematoma	25	316	”
3.	Polyneuritis	45	212	”
4.	Subarachnoid hemorrhage. Ruptured aneurysm	60	264	4.0
5.	Cerebral thrombosis and subarachnoid hemorrhage	90	170	trace*
6.	Cerebral thrombosis	90	154	2.5
7.	Head injury with cerebral laceration	90	50	3.0
8.	Subarachnoid hemorrhage. Ruptured aneurysm	120	54	10.8
9.	Brain tumor post operative	180	22.3	trace*
10.	Encephalitis	180	30	1.0

* Approximately 0.5 mg per 100 cc.

fluid, but that equilibrium was reached slowly. The extracellular fluid formed a reservoir out of which sucrose diffused very slowly, and in one instance its presence was demonstrated in fluid and plasma 97 hours after the material was injected, while in normal subjects negative results were obtained in a much shorter time. In the 7 patients studied 20 hours or more after the sugar was given the concentration of the sugar was higher in the abnormal fluid than in the plasma.

It seemed desirable to extend the study to the equilibrium between blood and spinal fluid, for (A) a previous experiment[9] had demonstrated the presence of sucrose in the spinal fluid of a child who died shortly after an injection of the sugar, and (B) a late rise in intracranial pressure, following the initial drop, had been observed in some patients to whom sucrose had been given intravenously. In spite of the low values for spinal fluid sucrose reported by others, it seemed possible that the spinal fluid might serve as a reservoir for the sugar since such an effect had been observed in edema, peritoneal, and pleural fluids.

The method used was similar to that employed by Hubbard and Anderson.[8] 25 g of sucrose, given as 50 cc of a 50% solution were injected intravenously into 10 patients suffering from various clinical conditions. Specimens of blood and fluid were withdrawn after various periods and analyzed for sucrose. In each instance duplicate specimens were fermented, one by a strain of *Escherichia coli* which fermented all common sugars, and the other by a strain which did not destroy sucrose, but did ferment all other sugars present in body fluids. After clarification by Somogyi's zinc method[10] sucrose was determined by the resorcinol method of Roe.[11] The method gave satisfactory recovery of sucrose added to plasma and to spinal fluid, and appeared to demonstrate successfully concentrations greater than about 0.5 mg per 100 cc.

The results are given in Table I. It is evident that sucrose was present in spinal fluid in very small amounts, and that only when a hemorrhage was present could the amount found be considered significant. Even then the concentrations were so low that the authors do not believe they could explain any secondary rise in intracranial pressure, even if they had persisted until all the sucrose had been excreted from the blood by the kidneys. The figures are probably influenced by a slow diffusion of sucrose through the spinal fluid after it had entered the canal, but it seems improbable that this factor is adequate to explain the great difference between these figures and the values reported by Hubbard and Anderson for other extracellular fluids. It seems certain that, in contrast with the ease with which sucrose passes out of the capillaries into accumulations of body fluid and is filtered through glomeruli, it normally passes from the blood into the spinal fluid in very small amounts.

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[9] Hubbard, R. S., and Terplan, K., *Arch. Path.*, 1935, **20**, 307.

[10] Somogyi, M., *J. Biol. Chem.*, 1930, **86**, 655.

[11] Roe, J. H., *J. Biol. Chem.*, 1934, **107**, 15.

Summary. After intravenous injection sucrose occurs in the spinal fluid in very low concentration. Even when hemorrhages into the central nervous system occur, the concentration does not approach that found in blood.

16939

Inhibition of Development of Sarcoma 180 by 4-Amino-N^{10}-Methyl Pteroylglutamic Acid.*

Alice E. Moore, C. Chester Stock, K. Sugiura, and C. P. Rhoads.
From the Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, New York City.

In view of the extensive developments among the anti-metabolites it was a natural consequence that cancer chemotherapy studies should be initiated with analogs of folic acid. Interest in the folic acid antagonists has been stimulated by reports of the effectiveness of Aminopterin in the treatment of leukemia (Farber).[1] In an intensive screening program during the past year a large number of analogs of folic acid and various related simple compounds have been studied against mouse leukemia[2] and various mouse and rat tumors with particular emphasis on the Crocker mouse Sarcoma 180. Thus far, one folic acid antagonist,[3] 4-Amino-N^{10}-methyl pteroylglutamic acid (A-Methopterin),[†] has been outstanding in its ability to inhibit the growth of Sarcoma 180. The preliminary results of the study are presented.

Materials and methods. The material was tested for ability to inhibit the growth of the Sarcoma 180 and also for its ability to damage a well established tumor.

Carefully selected tumors were cut into 2 mm cubes and implanted subcutaneously into the axillary region of groups of 5 weighed and marked white mice. In the inhibition tests, intraperitoneal injections of 0.5 cc of different doses of A-Methopterin were begun 24 hours later. In the therapeutic test, injections were begun after 3 to 7 days of tumor growth. In all instances, injections were given twice daily at 6-hour intervals for 7 consecutive days. If the animals showed definite signs of a toxic effect of the drug, administration was omitted until recovery had taken place. At the higher dosage (2.5-2.0 mg/kg/day) 3 or 4 omissions were often necessary. Below this level the drug was well tolerated.

Following the period of injections the animals were weighed and the size of their tumors determined by measurement in 2 diameters with calipers. The size of the tumors was then compared with those of the untreated controls and tumor growth was graded according to the following scheme: 1) Inconclusive, in groups of animals where 3 or more died, 2) No effect, when the largest diameter was ¾ or more of the diameter of the control tumors, 3) Moderate effect, when the diameter was ¼ to ¾ of the diameter of the control tumors, 4) Marked inhibition when the diameter was ¼ or less of that of the control tumors; actually,

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* We wish to acknowledge support of this study by funds from the American Cancer Society.

[1] Farber, S., Diamond, L. K., Mercer, R. D., Sylvester, R. F., Jr., and Wolff, J. A., *New England J. Med.*, 1948, **238**, 787.

[2] Burchenal, J. H., Burchenal, J. R., Kushida, M. N., Johnston, S. F., and Williams, B. S., *Cancer*, 1949, **2**, 113.

[3] Franklin, A. L., Belt, M., Stokstad, E. L. R., and Jukes, T. H., *J. Biol. Chem.*, 1949, **177**, 621.

[†] A-Methopterin is the name given to the compound by Lederle Laboratories, to whom we are indebted for supplies. The Calco Division, American Cyanamid Company also has provided a supply of this compound.

TABLE I.
Effect of Different Amounts of A-Methopterin on the Growth of the Sarcoma 180.

Dose in mg/kg/day	No. of groups of 5 mice	No. of mice	Deaths during inj. period		Effect on tumor*			
			No.	%	Inconclusive	Marked inhibition	Moderate effect	No effect
2.5	6	30	14	46.6	2*	4	0	0
2.0	15	75	14	18.6	2	9	4	0
1.5	31	155	11	7.1	0	16	11	4
1.0	8	40	2	5.0	0	1	4	3

* The tumor effects are expressed as the result obtained in each group of 5 mice. Thus, the numeral 2 indicates two groups of 5 mice each.

the volume of such a tumor would be 1/64 or less of the control if tumors may be considered spherical.

The animals were again weighed and their tumors measured at the end of the second week.

Results. Groups of 5 mice implanted with the Sarcoma 180 one day previously were injected intraperitoneally with daily doses of A-Methopterin at the following levels: 2.5 mg, 2.0 mg, 1.5 mg, and 1 mg/kg. Deaths due to the toxicity of the material were recorded during the 7-day period of injection. Tumor measurements were made at the end of the period and the effect of the substance on the tumor was evaluated. These data are summarized in Table I.

It is clear that A-Methopterin has a definite inhibitory effect on the growth of Sarcoma 180 at all the levels of drug above 1 mg/kg/day. The drug killed 46% and 18%, respectively, in the groups of mice receiving 2.5 and 2.0 mg/kg/day. At the lower dosages 7% and 5% of the mice receiving 1.5 and 1.0 mg died during the treatment. Male mice appeared to tolerate the drug better than female mice. The best results were obtained at the 1.5 mg dose where a definite effect was noted in almost all instances with little evidence of toxicity. The average weight loss of this group was 1.0 g as contrasted to a gain of 0.5 g for the controls. Fig. 1 shows the typical inhibition effect on the tumor of the injection of A-Methopterin at the level of 1.5 mg/kg/day.

The tumors have never been completely destroyed. When the injections are stopped, the small tumors grow normally.

When the tumors were allowed to grow for 3 to 7 days before the injections were begun, inhibition of growth was apparent in practically all instances. Of the 15 groups of mice receiving 2 mg/kg/day, inhibition was marked in 3 groups, moderate in 11 groups and absent in one group. Of the 24 groups receiving 1.5 mg/kg/day, inhibition was marked in 7, moderate in 16, and absent in one. The death rates for the 2 dosages were 12% and 16%, respectively. It is doubtful that these figures represent true toxicity deaths, because many of the tumors were so large at the time injections were initiated, that some of the mice probably died from the effects of tumor growth. The effect of A-Methopterin was definite although not as marked as in the groups of mice injected 24 hours after implantation.

In a few experiments a single injection of 50 mg/kg was administered when the tumor had grown one day. An inhibitory effect was evident when the tumors were measured a week later. In one group implanted 24 hours previously which received one injection of 50 mg/kg, small doses (0.5 mg/kg/day) were administered for 7 days. When the tumors were measured, they were very small but grew when the injections were stopped. The development of 7-day-old tumors was not affected by the injection of the single large dose.

Discussion. A-Methopterin has been shown to have a definite and consistent inhibitory effect on the growth of the transplantable mouse Sarcoma 180. In another report (Sugiura, Moore, Stock)[4] the folic acid antagonist, Aminopterin, was shown to have a

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[4] Sugiura, K., Moore, A., and Stock, C. C., *Cancer*, in press.

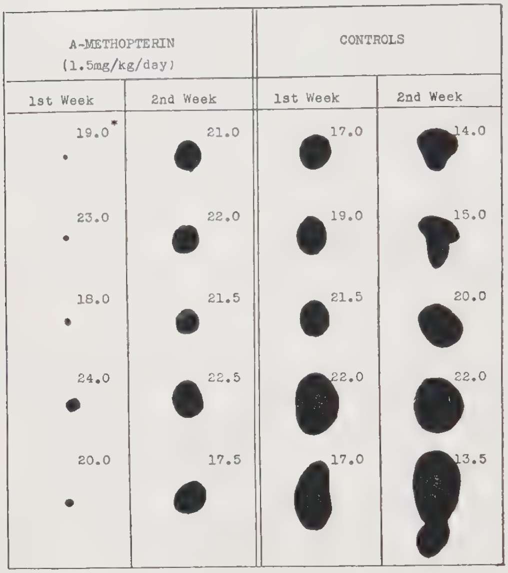

FIG. 1.
Area diagrams of tumors in mice treated with A-Methopterin compared with untreated controls.
* Weight of mouse in grams.

similar effect; however, marked inhibition could be demonstrated only when the drug was administered in amounts which killed a large per cent of the mice. A-Methopterin appears to exhibit a more favorable therapeutic index. It has been outstanding in its effect among numerous compounds which might interfere with the utilization of folic acid or possibly with the synthesis of nucleic acid. In fact, it has been the most effective compound of over 700 miscellaneous compounds that have been screened. It is therefore advanced as a compound worthy of further experimental study and for clinical trial in various types of cancer.

Summary. The compound 4-Amino-N^{10}-methyl pteroylglutamic acid, has shown the ability to inhibit the growth of the transplantable mouse Sarcoma 180 when administered intraperitoneally in concentrations of 1.5 mg/kg each day for 7 days. At this dosage very few mice died from drug toxicity. The inhibition is most apparent when the compound is administered early in the development of the tumor.

16940

Beneficial Effects of Liver on Growth and Survival in the Immature Hyperthyroid Mouse.*

B. H. ERSHOFF.

Emory W. Thurston Laboratories, Los Angeles, and Department of Biochemistry and Nutrition, University of Southern California, Los Angeles.

Available data indicate that desiccated whole liver will prolong survival and counteract the growth retardation of immature rats fed massive doses of thyroactive materials.[1-5] The protective factor is distinct from any of the known nutrients and is retained in the water-insoluble fraction of liver.[3-5] The present experiment was undertaken to determine the effects of liver and liver fractions on growth and survival in the immature hyperthyroid mouse.

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[1] Ershoff, B. H., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 500.

[2] Betheil, J. J., Wiebelhaus, V. D., and Lardy, H. A., *J. Nutrition*, 1947, **34**, 431.

[3] Ershoff, B. H., *Arch. Biochem.*, 1947, **15**, 365.

[4] Ershoff, B. H., *Exp. Med. Surg.*, 1948, **6**, 438.

[5] Ershoff, B. H., and McWilliams, H. B., *Science*, 1948, **108**, 632.

Procedure and results. One hundred and eighty-seven male mice of the Webster strain were selected for the present experiment at 21 to 23 days of age and an average weight of 10.7 g. The basal ration employed consisted of sucrose 53%, casein† 30%, cottonseed oil 10%, Sure's Salt Mixture No. 1[6] 5%, and cellulose‡ 2%. To each kg of the above diet were added the following synthetic vitamins: thiamine hydrochloride 20 mg, riboflavin 20 mg, pyridoxine hydrochloride 20 mg, nicotinic acid 60 mg, calcium pantothenate 60 mg, biotin 4 mg, folic acid 10 mg, p-aminobenzoic acid 200 mg, inositol 400 mg, 2-methylnaphthoquinone 10 mg, and choline chloride 2 g. Each mouse also received once weekly a vitamin A-D concentrate§ containing 25 U.S.P. units of vitamin A and 2.5 U.S.P. units of vitamin D together with 1.5 mg of α-tocopherol acetate. The following supplements were incorporated in the basal ration in place of an equal amount of sucrose: (1) 10% yeast‖ (2) 10% whole liver powder¶ (3) 10% extracted liver residue,** consisting of the coagulated, water-insoluble material remaining after the removal of the extractable water-soluble substances and (4) 2% of Wilson's liver concentrate 1-20,†† containing the water-extractable material of raw liver. Each of the above diets was in turn supplemented with U.S.P. desiccated thyroid,‡‡ thyroxin,§§ or iodinated casein,‖‖ added in place of an equal amount of sucrose. These were incorporated in the above rations at the following levels: thyroid, 0.5%; thyroxin, 50 mg/kg of diet, and iodinated casein, 0.2%. Animals were placed in metal cages with raised screen bottoms to prevent access to feces and were fed the above diets *ad lib.* Feeding was continued for 15 days or until death, whichever occurred sooner. Animals that died or lost weight during the first 5 days of feeding were discarded and not included in the tabulation of data. Results are summarized in Table I.

The findings indicate the response of the immature mouse is similar to that of the rat when fed massive doses of thyroactive materials. The addition of desiccated thyroid, thyroxin or iodinated casein to the basal ration resulted in a marked retardation of growth which was completely counteracted by the administration of whole liver powder or extracted liver residue. Liver concentrate 1-20 failed to promote growth in animals fed desiccated thyroid or iodinated casein; it did exert a growth-promoting effect, however, in animals fed thyroxin. Yeast was ineffective in promoting growth on any of the diets employed. Data on survival correspond to those obtained in the rat. Less than 50% of the mice fed the basal ration supplemented with desiccated thyroid, thyroxin or iodinated casein survived the experimental period of 15 days. The incidence of survival was significantly increased on diets containing whole liver powder or extracted liver residue. Yeast and liver concentrate 1-20 were considerably less active than whole liver or extracted liver residue in prolonging survival in the hyperthyroid mouse.

In the present experiment whole liver powder prolonged survival and counteracted the growth retardation of immature mice fed massive doses of desiccated thyroid, thyroxin, or iodinated casein. The protective factor(s) was apparently distinct from any of the known nutrients and was retained in the water-insoluble extracted liver residue. Bosshardt *et al.*[7] have recently found that the

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† Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[6] Sure, B., *J. Nutrition*, 1941, **22**, 449.

‡ Ruffex, Fisher Scientific Co., St. Louis, Mo.

§ Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per g.

‖ Brewers' Type Yeast No. 200, Anheuser-Busch, Inc., St. Louis, Mo.

¶ Whole Dried Liver Powder, Armour and Co., Chicago, Ill.

** Extracted Liver Residue, Wilson Laboratories, Chicago, Ill.

†† Liver Concentrate Powder 1-20, Wilson Laboratories, Chicago, Ill.

‡‡ Thyroid Powder, U.S.P., Armour and Co., Chicago, Ill.

§§ Thyroxin (Synthetic Cryst.), Roche-Organon, Inc., Nutley, N. J. The material was dissolved in .1 N NaOH, adjusted to pH of 8.0, and diluted to a volume containing 50 mg in 12.5 cc.

‖‖ Protamone, Cerophyl Laboratories, Kansas City, Mo.

TABLE I.
Comparative Effects of Liver and Yeast on Growth and Survival of Immature Mice Fed Massive Doses of Thyroactive Substances.

Supplement to basal ration	Thyroactive supplement	No. of animals	Initial body wt, g	Gain in body wt on following days of exp. 10th, g	Gain in body wt on following days of exp. 15th, g	% surviving*
0	0	12	10.6	6.2 (12)	8.1 (12)	100.0
0	Thyroid	15	10.6	2.6 (5)	5.4 (1)	6.7
10% yeast	”	10	10.7	2.9 (8)	5.2 (6)	60.0
10% whole liver powder	”	14	10.5	7.7 (14)	11.2 (13)	92.9
10% ext. liver residue	”	15	10.4	6.6 (15)	10.0 (15)	100.0
1% liver conc. 1-20	”	15	10.5	3.3 (9)	3.8 (6)	40.0
0	Iodinated casein	12	10.9	2.8 (10)	4.2 (4)	33.3
10% yeast	” ”	10	11.0	2.8 (10)	5.1 (6)	60.0
10% whole liver powder	” ”	12	10.7	8.4 (11)	11.9 (11)	91.7
10% ext. liver residue	” ”	9	11.1	7.3 (8)	9.7 (8)	88.9
1% liver conc. 1-20	” ”	8	11.1	2.0 (8)	3.6 (6)	75.0
0	Thyroxin	11	10.7	1.0 (10)	2.6 (5)	45.5
10% yeast	”	9	10.8	3.6 (6)	4.3 (6)	66.7
10% whole liver powder	”	11	10.8	5.3 (11)	8.4 (9)	81.8
10% ext. liver residue	”	12	10.6	8.9 (11)	12.8 (11)	91.7
1% liver conc. 1-20	”	12	10.9	6.4 (7)	9.8 (6)	50.0

The values in parentheses indicate the number of animals which survived and on which averages are based.

* Experimental period 15 days.

growth retardation of immature mice fed massive doses of iodinated casein (protamone) could be counteracted by the administration of Wilson's liver concentrate 1-20 and other water-soluble extracts of liver rich in "animal protein factor" or vitamin B_{12}. Under conditions of the present experiment, Wilson's liver concentrate 1-20 contained virtually no activity in regard to either growth or survival for immature mice fed massive doses of iodinated casein or desiccated thyroid. It did exert a growth-promoting effect, however, in immature mice fed thyroxin, although gain in body weight was less than that obtained with extracted liver residue. The cause of this discrepancy is not readily apparent. Yeast appears to be less beneficial to the hyperthyroid mouse than to the hyperthyroid rat. Previous findings indicate that yeast is as effective as whole liver powder in prolonging survival of immature rats fed massive doses of desiccated thyroid;[3] such does not appear to be the case for the immature thyroid-fed mouse.

Recent findings by Bosshardt *et al.*,[7] Register *et al.*,[8] and Nichol *et al.*,[9] indicate that requirements for vitamin B_{12} are increased in the hyperthyroid animal. Since liver concentrate 1-20 (which contains vitamin B_{12}) failed to counteract the growth retardation of immature mice fed massive doses of desiccated thyroid, thyroxin or iodinated casein while extracted liver residue was as potent as whole liver powder in this regard, it appears that extracted liver residue contains one or more factors other than vitamin B_{12} which are also required in increased amounts by the hyperthyroid mouse. These findings are in agreement with those previously reported for the rat.[4] When animals on purified rations are made hyperthyroid, a multiple dietary deficiency may be precipitated. Whether the first limiting factor will be a deficiency of vitamin B_{12}, of the antithyrotoxic factor(s) of extracted liver residue or of still other nutrients appears to depend on such factors as the pre-test dietary regime, the composition of the diet employed, bacterial synthesis or strain and species differences in nutritional requirements.

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[7] Bosshardt, D. K., Paul, W. J., O'Doherty, K., Huff, J. W., and Barnes, R. H., *J. Nutrition*, 1949, **37**, 21.

Summary. Whole liver powder prolonged survival and counteracted the growth retardation of immature mice fed massive doses of desiccated thyroid, thyroxin or iodinated casein. The protective factor(s) was retained in the water-insoluble fraction of liver.

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[9] Nichol, C. A., Dietrich, L. S., Cravens, W. W., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 40.

16941

An Evaluation of Respiratory Depression by Alveolar Gas Changes During Pentothal Sodium Anesthesia.*

RICHARD AMENT,† MITZI SUSKIND, AND HERMANN RAHN.

From the Department of Physiology and Vital Economics, University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.

Recent studies of intravenous anesthetic agents, notably pentothal sodium, have placed renewed emphasis on quantitative analyses of physiological changes occurring during anesthesia. The work of Gruber,[1] Gruhzit, Dox, Rowe and Dodd,[2] Beecher and Moyer,[3,4] Moyer,[5] and most recently McCann,[6] contain pneumographic tracings which illustrate the characteristic respiratory patterns produced by intravenous anesthetic agents. Beecher and Moyer[4] have correlated these qualitative studies with minute volume determinations and intermittent blood levels of oxygen and carbon dioxide. Etsten and Himwich[7,8] intensively investigated the reflex responses under pentothal anesthesia and have set forth criteria for determining the stages of pentothal anesthesia. Previous studies have employed

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* Work done under contract with the Air Materiel Command, Wright Field.

† Present address: Department of Anesthesia, Bellevue Hospital, New York.

[1] Gruber, C. M., *J. Pharm. and Exp. Therap.*, 1937, **60**, 143.

[2] Gruhzit, O. M., Dox, A. W., Rowe, L. W., and Dodd, M. C., *J. Pharm. and Exp. Therap.*, 1937, **60**, 125.

[3] Beecher, H. K., and Moyer, C. A., *J. Clin. Invest.*, 1941, **20**, 549.

[4] Moyer, C. A., and Beecher, H. K., *J. Clin. Invest.*, 1942, **21**, 429.

[5] Moyer, C. A., *J. Thoracic Surg.*, 1941, **11**, 131.

[6] McCann, J. C., *Current Res. in Anesth.-Anal.*, 1947, **26**, 89.

[7] Himwich, H. E., and Etsten, B., *J. Nerv. and Mental Disease*, 1946, **104**, 407.

[8] Etsten, B., and Himwich, H. E., *Anesthesiology*, 1946, **7**, 536.

the measurement of total ventilation volume as an index of respiratory depression or stimulation. As will be discussed below such measurements may lead to erroneous conclusions when applied quantitatively. Instead, it is here proposed to describe the degree of the effective ventilation in terms of the alveolar gas concentration only, which makes the consideration of volume measurements unnecessary. This method has the further advantage that the alveolar gas concentrations reflect the gas pressures of the arterial blood if corrected for the alveolar-arterial gradient. Suskind[9] has recently shown that during anesthesia this gradient, at least for CO_2, is nonexistent. Thus changes which occur in the pCO_2 of the alveolar air allow one to calculate changes in the arterial pH if the plasma bicarbonates are known.

Method. Alveolar air was automatically sampled by a simple device described by Rahn and Otis[10] and continuously analyzed as previously reported.[11] Readings were made at one minute intervals and the respiratory quotient calculated from the alveolar air equation (Fenn, Rahn, and Otis).[12]

Ventilation volume. The air was expired through a gas meter. Each passing of 457 cc of gas produced an electrical contact which closed the circuit of a magnetic writing pen recording on a constant-speed paper tape.

Frequency of respiration was recorded on the same tape by another pen. The slight negative mask pressure during each inspiration activated a sensitive aneroid which in turn closed the electrical circuit.

Three dogs each weighing approximately 23 kilos were fitted with individually constructed skin-tight masks. After an initial training period alveolar air could be sampled in the unanesthetized animal. Pentothal sodium was then injected into the saphenous vein and all the respiratory phenomena were recorded without interruption. After losing consciousness the dogs were placed in the lateral recumbent position with the head in hyperextension for the remainder of the experiment. Specific attention was placed on the maintenance of an adequate, unobstructed airway by position of the head.

Alveolar gas analyses were also performed in the operating pavilion on 9 patients undergoing elective operative procedures under pentothal anesthesia administered by an intermittent injection technic.

Results. 1. Single injection of 0.5 g of pentothal sodium in dogs.

Three dogs were each given a single injection of 0.5 g of pentothal over a 2-5 minute period with mask in place while continuous analyses were being carried out. Observations were continued until the animal had

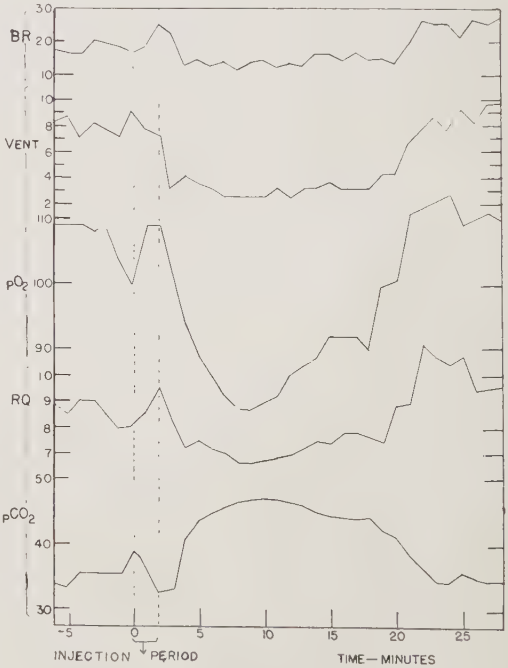

Fig. 1.

Respiratory responses of dog S to 0.5 g pentothal sodium. Injection occurred between 0 and 2 minutes. BR, breaths per minute; Vent, liter per minute expired at B.T.P.S. (body temperature, pressure and saturated): pO_2 and pCO_2 mm Hg alveolar pressure of the gases; R.Q., alveolar respiratory quotient.

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[9] Suskind, M., unpublished.

[10] Rahn, H., and Otis, A. B., *J. Applied Physiol.*, 1949, **1**, No. 10.

[11] Rahn, H., Mohney, J., Otis, A. B., and Fenn, W. O., *J. Aviation Med.*, 1946, **17**, 173.

[12] Fenn, W. O., Rahn, H., and Otis, A. B., *Am. J. Physiol.*, 1946, **146**, 637.

recovered to the point of restlessness, or for a period of 30 to 55 minutes. Maximum respiratory depression was reached in approximately 10 minutes and a gradual return to normal respiratory values followed. Signs of awakening and return of reflexes occurred as the respiratory picture of the animals approached control levels.

A typical record is shown in Fig. 1. It can be seen that the period of maximum depression is attained about 10 minutes after injection. This depression period is characterized by the lowest ventilation volume with its concomitant low pO_2, R.Q. and high pCO_2. The last period of recovery is associated with relative hyperventilation and an R.Q. above normal.

2. Intermittent injection of fractional doses of pentothal sodium.

Two dogs were given intermittent injections of approximately 0.25 g of pentothal sodium until a total of 1 g was administered. These experiments were carried on in precisely the same manner except that the recordings extended over a period of 140 minutes. The same general pattern of Fig. 1 and 2 repeated itself following each intermittent injection.

3. Intermittent injection of pentothal sodium in patients undergoing elective operative procedures.

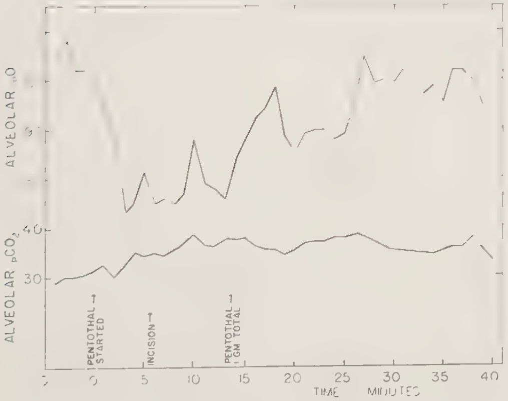

Fig. 2.

Alveolar pO_2 and pCO_2 recorded continuously in a patient before, during and after receiving pentothal sodium intermittently over a 14-minute period. Total dose 1 g. The fluctuations, particularly in the pO_2 level, arise from reflex hyperventilation.

Nine patients undergoing operative procedures were observed. Attempts to obtain control levels of alveolar air in the immediate preoperative period were not totally successful because of the apprehensive state of mind of the patient and the disturbances caused by preoperative medication. During light pentothal anesthesia operative stimulation always produced marked reflex hyperventilation which is reflected in the recorded values (Fig. 2). However, these responses are superimposed upon the respiratory pattern described above as characteristic of pentothal anesthesia in dogs.

Discussion. The question arises of a suitable quantitative criterion for the measurement of respiratory depression. If the total ventilation is used as an index one may make two errors. The first error is produced by changes in the oxygen consumption from the resting, conscious state to the anesthetized state. Thus a reduction of 30% of ventilation is without effect upon the alveolar gas concentration if likewise the oxygen consumption drops by the same amount. The second error is encountered when the breathing rate alters the dead space ventilation. For example a total ventilation of 6 liters per minute may break down into 4 liters alveolar ventilation and 2 liters dead space ventilation (10 respirations of a 200 cc dead space). The same total ventilation can be achieved by 20 respirations of the same dead space and a 2 liter alveolar ventilation. If the former produces a normal alveolar gas composition the latter situation would produce hypoxia and severe acidosis.

Thus unless these entities are known or can be measured the total ventilation may be a poor quantitative index of respiratory depression or stimulation. This is particularly so if one is interested in the effects of the altered ventilation upon the blood and tissue gas tensions.

The next best alternative is to measure *absolute alveolar ventilation* which eliminates the dead space ventilation but is dependent upon the oxygen consumption and for this reason is still unsatisfactory.[12] If respiratory depression or stimulation must be expressed

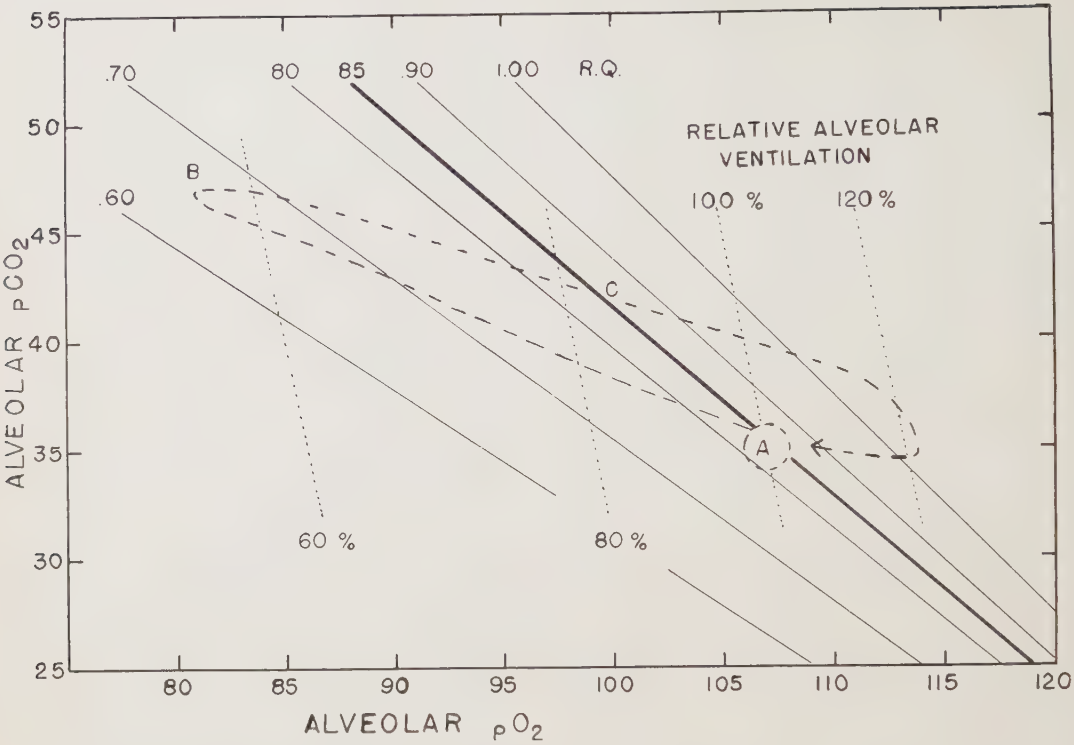

FIG. 3.

Typical alveolar pathway of dog S to single injection of pentothal sodium indicating the simultaneous alveolar pO_2, pCO_2, R.Q., and relative alveolar ventilation. Point A is the average, unanesthetized level prior to injection. Region B indicates the period of deepest depression, region B-C the period of compensation, and C-A the period of recovery characterized by high R.Q. For details see discussion.

in terms of ventilation volume it probably can be most satisfactorily done by calculating the *relative alveolar ventilation* from the alveolar gas concentration. This new derivation is discussed below.

On the other hand the difficulties in the foregoing discussion may be entirely avoided if respiratory depression or stimulation is measured in terms of the deviation it induces from the normal alveolar CO_2 and O_2. Thus the net result of the effective ventilation is expressed without involving the measurement of a ventilation volume. In Fig. 1 the various recorded values are plotted against time. In Fig. 3 the alveolar O_2 and CO_2 are plotted against each other and the whole period of anesthesia is represented by a point moving in a clockwise direction. Each point on such a graph simultaneously determines the alveolar respiratory quotient as well as the relative ventilation index. The iso-R.Q. lines are straight and originate at the inspired O_2 tension while the iso-ventilation lines have a negative slope of .209 when air is inspired.[12] The advantage of this type of graph is that it allows the simultaneous visualization of the various components of respiration.

Point A in Fig. 3 is the average alveolar air composition of the unanesthetized dog just prior to the injection of pentothal. The R.Q. is .85 and the alveolar ventilation is assumed to be normal or 100%. Following the injection, respiration is depressed. This is seen by a rapid fall in the alveolar pO_2 and R.Q. and a slower rise of the pCO_2 until point B is reached. The curve from A to B is very constant in dog and man and has been designated as the "respiratory acidosis pathway" by Rahn and Otis.[10] It is reproducible from time to time and has been observed whenever the alveolar ventilation is reduced by dead space breathing, anesthesia or voluntary apnea.[10,13]

Point B indicates the deepest depression reached about 10 minutes after the injection and represents the greatest retention of metabolic CO_2 as indicated by the low R.Q. value. Since Suskind[9] has recently shown in this laboratory that there is no significant difference in the anesthetized dog between the arterial and alveolar pCO_2 one may estimate the changes in arterial pH accompanying the pCO_2 rise. Assuming a normal base bicarbonate level of the plasma the Henderson-Hasselbalch equation indicates a pH drop of .12 units between point A and B. Unpublished studies with standard doses of nembutal show much greater depression yielding CO_2 and O_2 values of 50 and 60 mmHg respectively.

This initial respiratory depression is followed by a period of compensation. The CO_2 stores of blood and tissues are slowly adjusted to the new alveolar CO_2 tension and as the rate of accumulation of CO_2 decreases, the R.Q. returns to its original value (Point C). However, the new steady state is not maintained. Throughout the period of compensation, the anesthetic is wearing off, and the sensitivity of the respiratory system to CO_2 is increasing. Thus we see during the final stages of recovery (region C-A) a relative hyperventilation which rids the blood and tissues of the recently acquired CO_2 stores and is manifested by an R.Q. above .85. The 3 respiratory phases described here, depression A-B, compensation B-C, and recovery C-A, all grade into one another and last approximately 10 minutes each. With complete recovery the CO_2 tension in blood, tissues and alveoli are readjusted to the original value of point A and the steady state with a normal R.Q. is observed.

At this time the dog becomes restless, and when an additional injection is given the whole cycle repeats itself. This was accomplished 3 times in succession in one dog and twice in another, the total period of anesthesia lasting over a period of 2 hours. This alveolar pathway cycle is typical not only for anesthesia but also in conscious man when the alveolar ventilation is reduced by various artificial means.

Should it be desirable to express the drug action in terms of a ventilation unit this can be done by assuming that the ventilation is normal (100%) only when it maintains an alveolar concentration of point A, the control value during the conscious state. This unit is called the *relative alveolar ventilation,* which has the advantage that it makes ventilation independent of changes in metabolic rate and thus offers a better physiological comparison for fluctuating metabolic rates as encountered in anesthetic studies.

The *relative alveolar ventilation* of point A is equal to $\frac{k.\ R.Q.\ 100}{\text{alv. } pCO_2}$ where k is a constant yielding a ventilation of 100% when the values of point A are substituted. (This equation is derived from the alveolar ventilation equation, Fenn[12]). The constant for our example is 41.2. When lines of equal "relative alveolar ventilation" are plotted they form straight lines as indicated in Fig. 3.

It can be seen that the deepest respiratory depression is obtained when the alveolar ventilation is 58% of the ventilation required to maintain the alveolar air composition at point A. Recovery, on the other hand, is associated with a relative hyperventilation reaching a maximal value just over 120%. Fig. 1, however, indicates a much larger relative change in the *total ventilation,* (from 100% to 38%) and is therefore misleading unless the marked drop in oxygen consumption and breathing rate is properly evaluated.

It is obvious from the data that respiratory stimulation or depression is a continuously changing state. This makes a quantitative comparison difficult unless some particular phase of the anesthesia cycle is employed as reference. Since *total ventilation* as an index may be quite misleading it is here suggested that the deviation from the normal alveolar pCO_2, R.Q. and/or *relative alveolar ventilation* be used as a quantitative and physiologically comparable approach to studies of res-

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[13] Otis, A. B., Rahn, H., and Fenn, W. O., *Am. J. Physiol.,* 1948, **152**, 674.

piratory depression or stimulation until continuous arterial blood gas analyses become more practical.

Summary. 1. The alveolar O_2, CO_2 tension and R.Q., minute volumes and respiratory rates have been recorded continuously in animals and man under pentothal sodium anesthesia administered by single and intermittent injection.

2. When the alveolar gas concentrations are plotted with the aid of a CO_2-O_2 diagram a typical anesthesia pattern can be shown indicating the regions of depression, compensation and recovery.

3. With intermittent injections of pentothal sodium this typical pattern is repeated.

4. The theoretical significance of this respiratory pattern and its utilization for analysis of quantitative changes in respiration in the study of anesthetic agents is discussed.

16942

A Technic for the Recovery of Viruses from Crude Feces.

Herbert R. Morgan.*

From the Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor, Mich.

During the course of studies on the isolation of psittacosis and other viruses from feces, an attempt was made to develop a method, which would allow for direct inoculation of a crude fecal suspension into eggs, for the recovery of a variety of viruses by the use of anti-bacterial agents. Several different test agents were used to determine the applicability of the technic to known viruses in the hope that it might prove useful in the isolation of as yet unknown viruses from the gastrointestinal tract of man and animals. Since penicillin and the sulfonamides are known to inhibit certain viruses of the psittacosis-lymphogranuloma venereum group, they were eliminated from consideration.[1,2] Streptomycin has been shown to have no effect on them[1] nor on several other virus agents[3-6] so that it was useful for study. However, streptomycin alone is not efficacious against all gram positive organisms[7,8] so that it was necessary to find another agent to aid in the suppresion of the gram positive organisms in the feces.

The anti-bacterial agents tested were streptomycin, tyrothrycin in alcoholic solution, tyrothrycin in propylene glycol solution and crystal violet.

Since specimens are frequently submitted for virus isolation studies from patients receiving sulfonamide and/or penicillin therapy, it was of interest to determine whether psittacosis virus strains, known to be susceptible to their action, could be recovered from virus-fecal suspensions containing sulfadiazine or penicillin by the use of antagonistic or inactivating compounds such as p-aminobenzoic acid or cysteine hydrochloride.

The yolk sac route was chosen for the

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* Part of this work was carried out under the tenure of a Senior Fellowship in Medical Sciences of the National Research Council while working at the Thorndike Memorial Laboratory, Boston City Hospital.

[1] Early, R. L., and Morgan, H. R., *J. Immunol.*, 1946, **53**, 151.

[2] Meiklejohn, G., Wagner, J. C., and Beveridge, G. W., *J. Immunol.*, 1946, **54**, 1.

[3] Morgan, H. R., and Wiseman, R., Proc. Soc. Exp. Biol. and Med., 1946, **62**, 130.

[4] Florman, A. L., Weiss, A. B., and Council, F. E., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 16.

[5] Lowell, F. C., and Buckingham, M., Proc. Soc. Exp. Biol. and Med., 1946, **62**, 228.

[6] Henle, G., Henle, W., Wendell, K. K., and Rosenberg, P., *J. Exp. Med.*, 1948, **88**, 223.

[7] Hodges, J. H., *Science*, 1946, **104**, 460.

[8] Rose, H. M., Pearce, E., and Molloy, E., Proc. Soc. Exp. Biol. and Med., 1946, **62**, 124.

inoculation of eggs since (a) it allows for inoculation of a large volume of material and (b) it has been shown to be the route of choice for the inoculation of psittacosis virus as well as an excellent method for the cultivation of a wide variety of viruses.[9–11]

Three viruses were selected as models for this study: psittacosis (strain 6 BC), influenza A (PR8) and mumps.

Material and methods. A 10% suspension of human feces in infusion broth was prepared by homogenization in a Waring blendor. Large sized particles were removed by centrifugation at 800 rpm for 3 minutes. The suspension had a bacterial plate count of 2.1×10^7 organisms per ml. An aliquot of the fecal suspension was sterilized in the autoclave. To separate aliquots of the fresh fecal suspension and of the sterile fecal suspension, an active preparation of one virus was added to give a virus dilution of 1:10. Each different suspension was placed in a separate series of glass ampoules, sealed and stored in the dry icebox. The virus preparations added were: (a) a suspension of yolk sacs infected with psittacosis virus, or (b) allantoic fluid infected with PR8 influenza virus, or (c) allantoic fluid infected with mumps virus.

In testing each of the viruses with a diluent containing different types and amounts of inhibiting agents, serial 10 fold dilutions of the following were prepared:

(a) Virus-sterile feces diluted in plain beef heart infusion broth

(b) Virus-sterile feces diluted in beef heart infusion broth containing the antibacterial agents

(c) Virus-fresh feces diluted in plain beef heart infusion broth

(d) Virus-fresh feces diluted in beef heart infusion broth containing the antibacterial agents

The dilutions were allowed to stand at room temperature (*i.e.* 20–24°C) for 1-2 hours following which 0.25 ml amounts of each were injected into the yolk sacs of 12 embryonated eggs 6 or 7 days old. The eggs were placed in an incubator at 35°C and were candled daily. Eggs inoculated with 10 fold dilutions of the virus + fresh feces in plain broth up through 10^{-6} died within 24-72 hours with gross evidence of contamination while eggs inoculated with the suspension diluted in broth containing the inhibitors died in from 2 to 10 days depending on the particular virus and the degree of its dilution. As the latter group of eggs died: (a) smears were made of the yolk sacs, stained and examined to detect bacteria, and, in the case of eggs injected with psittacosis virus, were stained by Machiavello's method to demonstrate the typical elementary bodies, (b) in some instances, the yolk sacs were cultured on blood agar plates and in thioglycollate broth to test the reliability of the smears as an indicator of bacterial contamination, (c) in the case of the eggs inoculated with suspensions containing influenza or mumps viruses, the yolk, allantoic and amniotic fluids of the egg were tested for their ability to agglutinate chicken red cells as an index of the presence of virus.

As eggs in the group inoculated with the virus-sterile feces suspension diluted in plain broth or broth containing the bacteriostatic agents died, they were tested as noted above for the presence of the specific virus injected. These two groups served as controls for any possible deleterious action of the bacteriostatic agents on the virus itself.

In order to see whether virus could be recovered from specimens containing penicillin or sulfonamides, the following experiment was carried out: (a) 1000 U penicillin were injected into each egg of a series along with the suspension of the virus in sterile feces. Then 15 mg of cysteine hydrochloride[12] were injected into different groups of these eggs at intervals up to 24 hours. (b) A second series of eggs was injected with a suspension

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[9] Morgan, H. R., Early, R. L., and McClain, M. E., *J. Inf. Dis.*, 1946, **79**, 278.

[10] Morgan, H. R., and Finland, M., Proc. Soc. Exp. Biol. and Med., 1948, **68**, 618.

[11] Beveridge, W. I. B., and Burnet, F. M., Special Report Series No. 256, Medical Research Council, His Majesty's Stationery Office, London, 1946.

[12] Chow, B. F., and McKee, C. M., Proc. Soc. Exp. Biol. and Med., 1945, **58**, 175.

of virus in sterile feces along with 10 mg of sulfadiazine. At intervals up to 24 hours, 1 mg of p-aminobenzoic acid[13,14] was injected into different groups of these eggs. In all instances, the eggs died after incubation at 35°C for several days. Smears of the yolk sacs were stained and examined for the presence of psittacosis virus elementary bodies.

Results. Using a diluent of beef heart infusion broth containing 10 mg of streptomycin and 2 mg of tyrothrycin per ml (containing 1% glycerine to give a stable suspension of the alcoholic solution of tyrothrycin added to broth), it was possible to recover the viruses of psittacosis, influenza or mumps which had been added to a fresh suspension of feces without any evidence that the bacteriostatic agents used in the diluent for the suppression of bacterial contaminants had any deleterious effect on the virus. In fact, in many instances, even the eggs injected with a 10^{-1} dilution of the fecal suspension of virus (10^{-2} dilution of virus infected fluid) could be shown to contain virus free of detectable bacteria even though this dilution was shown to contain at least 2.1×10^6 aerobic bacteria per ml. High dilutions (*i.e.* 10^{-2} or 10^{-3}) almost always gave virus free from bacterial contaminants. This technic was found useful in the isolation of psittacosis virus from the ground-up intestines and feces of mice infected with the virus by the intravenous route.

In some experiments, a solution of tyrothrycin in propylene glycol was used but this solvent was found to inhibit the growth of the viruses. Therefore, the alcoholic solution was used subsequently with the addition of 1% glycerine to stabilize its suspension in broth.

It is important to point out that tyrothrycin can be used as a bacteriostatic agent in materials to be tested in eggs only if the yolk sac route of inoculation is used since it is toxic in 0.2 mg amounts in the allantoic or amniotic sacs.

Crystal violet in amounts up to 0.25 mg was tolerated when injected into the yolk sac of embryonated eggs but was not effective in controlling the gram positive bacteria in fecal suspensions tested.

It was found that the injection of (a) 15 mg of cysteine hydrochloride or (b) 1 mg of p-aminobenzoic acid into the yolk sac any time up to 24 hours after the injection of as much as (a) 1000 U of penicillin or (b) 10 mg of sulfadiazine respectively in the virus-feces inoculum would permit the recovery of psittacosis virus (strain 6 BC) which was known to be sensitive to 250 U of penicillin[1] or 0.5 mg of sulfadiazine.[14]

Summary. Using an infusion broth diluent containing 10 mg streptomycin and 2 mg tyrothrycin per ml, it was possible, by injection into the yolk sacs of 6-7-day-old embryonated eggs, to recover free from bacteria: psittacosis, influenza or mumps viruses from suspensions of fresh feces to which they had been added. The diluent had no apparent injurious effect on these viruses.

P-aminobenzoic acid was found to counteract the inhibitory action of sulfadiazine allowing for the recovery of psittacosis virus when the virus was suspended in materials containing the sulfonamide and injected into eggs. The injection of the p-aminobenzoic acid could be delayed up to 24 hours when injected in 1 mg amounts after a virus suspension containing 10 mg of sulfadiazine. In a similar series of experiments it was found that 15 mg cysteine hydrochloride would overcome the inhibitory action of 1000 U penicillin on psittacosis virus when given as long as 24 hours after the virus suspension containing this antibiotic. These antagonistic compounds made it possible to recover psittacosis virus from materials containing large quantities of sulfadiazine or penicillin in spite of the fact that this strain of psittacosis virus is known to be susceptible to much smaller quantities of each of these therapeutic agents.

This technic was useful in the isolation of virus from the intestines of mice infected with psittacosis virus and may find application in the isolation of viruses from the gastrointestinal tract of other animals or man.

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[13] Morgan, H. R., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 29.

[14] Morgan, H. R., *J. Exp. Med.*, 1948, **88**, 285.

16943

Lupulon—An Antibiotic Extracted from the Strobiles of *Humulus lupulus*.*†

A. J. Salle, Gregory J. Jann, and Michael Ordanik.
From the Department of Bacteriology, University of California, Los Angeles.

Lupulon is a nearly white, crystalline substance obtained by fractionating the resins extracted with methanol and other solvents from the strobiles of the hop *Humulus lupulus*.

According to Taylor and Millidge[1] lupulon possesses the following properties: It has a melting point of 92°C, is unsaturated, adds bromine, decolorizes permanganate, and has the properties of a keto-enol.

Walker and Parker[2] reported that the hop resins exhibited antibacterial properties. Shimwell[3] found that lupulon inhibited the growth of Gram-positive bacteria but had no effect on Gram-negative organisms.

The present communication is concerned with a study of the bacteriologic and mycologic properties of lupulon *in vitro* and its use in the treatment of bacterial infections in mice.

Materials and methods. A solution of lupulon was prepared by dissolving one gram in 25 ml of propylene glycol, heating slightly, then adding sufficient distilled water to make a 1:500 opalescent emulsion of the resinous antibiotic.

The bactericidal (with the exception of *Mycobacterium tuberculosis*) and fungicidal spectra were determined by the penicylinder method using a medium having the following composition: Sodium chloride (NaCl), 5 g; beef extract (Bacto), 5 g; peptone (Bacto), 10 g; agar (Bacto), 20 g; and distilled water, to make 1000 ml. The medium was adjusted

TABLE I.
Effect of Lupulon on a Number of Bacteria by the Penicylinder Method.

Gram-positive bacteria 1:10,000 dilution of lupulon	Results	Gram-negative bacteria 1:500 dilution of lupulon	Results
Micrococcus lysodeikticus	—	*Aerobacter aerogenes*	+
'' *pyogenes* var. *aureus*	—	*Alcaligenes faecalis*	+
'' *ureae*	—	'' *viscosus*	+
Bacillus anthracis	—	*Escherichia coli*	+
'' *megatherium*	—	'' *coli* var. *communior*	+
'' *subtilis*	—	*Klebsiella pneumoniae*	+
Gaffkya tetragena	—	*Proteus vulgaris*	+
Rhodococcus roseus	—	*Pseudomonas aeruginosa*	+
Sarcina lutea	—	'' *synxantha*	+
'' *ureae*	—	*Salmonella enteritidis*	+
Streptococcus faecalis	—	'' *paratyphi A*	+
'' *lactis*	—	'' *schottmuelleri*	+
		'' *typhosa*	+
		Serratia marcescens	+
		Shigella ambigua	+
		'' *dysenteriae*	+
		'' *paradysenteriae*	+
		'' *sonnei*	+

+ = growth; — = no growth.

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† The lupulon used in these experiments was kindly supplied by the Western Regional Research Laboratory, U. S. Department of Agriculture, Albany, Calif.

[1] Taylor, T. W. J., and Millidge, A. F., Richter's Organic Chemistry, Vol. II, Nordeman Publishing Co., Inc., New York, 1939.

[2] Walker, T. K., and Parker, A., *J. Inst. Brew.*, 1937, **34**, 17.

[3] Shimwell, J. L., *J. Inst. Brew.*, 1937, **34**, 111, 191.

TABLE II.
Effect of Lupulon on a Group of Higher Organisms by the Penicylinder Method.

1:500 dilution of lupulon	Results
Aspergillus flavus	+
” *niger*	+
Candida albicans	+
Cryptococcus neoformans	+
Discomyces mexicanus	+
Epicoccum nigrum	+
Microsporum lanosum	+
Nocardia asteroides	+
” *madurae*	+
Penicillium glabrum	+
Rhizopus nigricans	+
Streptomyces pelletieri	+
Trichoderma köningii	+
Trichophyton gypseum	+

+ = growth.

to pH 7.0 and sterilized at 15 lb pressure for 30 minutes.

Proskauer and Beck's medium was used for the growth of *M. tuberculosis*. This is a liquid medium having the following composition: Asparagine, 5 g; potassium acid phosphate (KH_2PO_4), 5 g; potassium sulfate (K_2SO_4), 0.6 g; magnesium citrate soluble ($MgHC_6H_5O_7 \cdot 5H_2O$), 2.5 g; glycerol, 25 g; and distilled water, to make 1000 ml.

Results. The action of lupulon on a number of Gram-positive and Gram-negative bacteria by the penicylinder method is given in Table I. It may be seen that a 1:10,000 dilution of the antibiotic was effective against Gram-positive bacteria but displayed little or no activity against Gram-negative organisms even when used in a concentration as high as 1:500.

In Table II are given the results of lupulon on a group of higher organisms. The antibiotic produced no apparent effect when used in a 1:500 dilution.

Lupulon was tested against several organisms in the presence of 10% horse serum[‡] (Table III). The results show that lupulon exhibited no antibiotic effect when tested in the presence of this agent.

Eight mice were injected with 0.1 cc of a virulent broth culture of *Streptococcus pyogenes*. Four of the mice were treated with lupulon and 4 were used as controls. Doses of 2 mg of the antibiotic injected intraperitoneally every 3 hours failed to produce any demonstrable effect on the outcome of the infection. All animals were dead after 24 hours. It may be concluded that the antibiotic was completely inactivated *in vivo*.

According to Taylor and Millidge[1] lupulon has the structural formula shown in (A), Fig. 1. Lupulon may exist in any of the 3 forms shown but forms (B) and (C) probably predominate because they contain more highly conjugated systems and hence have more resonance of stabilization than (A).

Lupulon is slowly inactivated when in solution. The effect of a freshly prepared and an old solution of lupulon on *M. tuberculosis* H37Rv is shown in Table IV. A freshly prepared solution is considerably more effective than one 10 days old.

Inactivation on standing is compatible with the general instability of the molecule. The side chain in position 5 contains an allylic

TABLE III.
Effect of Lupulon on *Micrococcus pyogenes* var. *aureus* and *Mycobacterium tuberculosis* H37Rv in the Presence and Absence of Horse Serum.

Dilution of lupulon	*M. pyogenes* var. *aureus*		Dilution of lupulon	*M. tuberculosis*	
	With 10% serum	Without serum		With 10% serum	Without serum
1:10 × 1000	+	—	1:10 × 1000	+	—
1:20 ”	+	—	1:20 ”	+	—
1:25 ”	+	—	1:60 ”	+	—
1:33 ”	+	—	1:100 ”	+	—
1:60 ”	+	+	1:160 ”	+	—
1:100 ”	+	+	1:200 ”	+	—
1:200 ”	+	+	1:300 ”	+	+
1:300 ”	+	+	1:400 ”	+	+
Control	+	+	Control	+	+

+ = growth; — = no growth.

[‡] The horse serum used in these experiments was kindly supplied by the Cutter Laboratories, Berkeley, Calif.

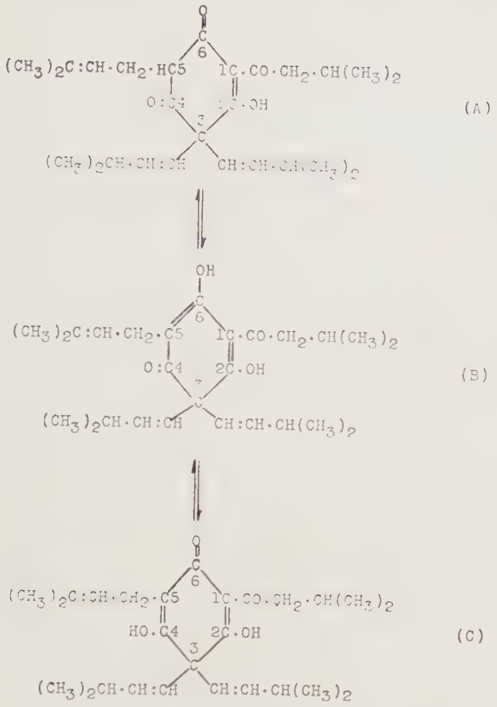

FIG. 1.

Lupulon may exist in any of the 3 forms shown above but probably (B) and (C) predominate.

methylene group in an extremely unstable arrangement. The double bonds in the 2 side chains in position 3 are susceptible to oxidation by air. The semi-aromatic system itself is, in general, an unstable configuration.

One would expect the hydroxyl in position 6 of tautomer (B) or the hydroxyl group in position 4 of tautomer (C) to acetylate under normal conditions. The hydroxyl in position 2 could be acetylated only with difficulty because of chelation with the carbonyl group on the one hand, and the hindrance due to the alkyl group in position 3 on the other.

Summary and Conclusions. Lupulon, an antibiotic extracted from hop resins, was found to be effective against Gram-positive bacteria *in vitro* but inactive against Gram-negative organisms. The antibiotic had no demonstrable effect on a number of pathogenic and nonpathogenic molds and actinomycetes.

Lupulon displayed no activity in the presence of horse serum. In the absence of serum the antibiotic exhibited its greatest activity against *M. tuberculosis* H37Rv. This may have been due to the fact that the tubercle bacillus was cultivated in a mineral medium with only an appreciable amount of interfering organic matter whereas the other organisms were grown in a medium containing peptone. The addition of serum to both media completely inactivated the antibiotic.

Lupulon is slowly inactivated in the presence of air and more rapidly in the presence of serum. Because of its inactivation by serum, the antibiotic is not suitable for clinical administration.

TABLE IV.
Effect of a Freshly Prepared and an Old Solution of Lupulon on *Mycobacterium tuberculosis* H37Rv.

Dilution of lupulon	Freshly prepared solution	Solution 10 days old
1:60 × 1000	—	—
1:80 ”	—	—
1:100 ”	—	+
1:140 ”	—	+
1:180 ”	—	+
1:200 ”	—	+
1:300 ”	—	+
Control	+	+

+ = growth; — = no growth.

16944

Effect of Autonomic Blocking Agents on Sweat Secretion in Cat.

HARRY D. PATTON. (Introduced by L. D. Carlson.)

From the Department of Physiology and Biophysics, University of Washington School of Medicine, Seattle, Wash.

Sweat glands, although innervated by sympathetic postganglionic fibers, are excited by parasympathomimetic drugs (acetylcholine, mecholyl) and blocked by atropine. Dale and Feldberg[1] found that eserinized perfusates collected from the cat's foot during stimulation of the sacral sympathetic ganglia gave positive pharmacological tests for an acetylcholine-like substance. Perfusates collected during stimulation were inactive, if the sweat glands were excluded from the circulation by ligating the base of the footpad. The sudomotor system was thus shown to be anatomically sympathetic, but pharmacologically parasympathetic (cholinergic).

A recent study by Haimovici[2] suggests, in addition, an adrenergic component of sudomotor innervation. He points out that ephedrine, benzedrine and neosynephrine, all sympathomimetic drugs, evoke moderate sweating in man. Using a qualitative colorimetric method for detecting sweat secretion, he noted depression of both spontaneous and neosynephrine-induced sweating in man after injection of Dibenamine (N, N-dibenzyl-β-chlorethylamine hydrochloride), although mecholyl still induced profuse sweating. Since numerous studies indicate that Dibenamine[3] is a specific adrenolytic and sympatholytic drug, he concludes that suppression of sweating by Dibenamine indicates, in addition to the known cholinergic innervation, an "adrenergic component in the nervous mechanism of sweating in man."

In the present experiments the effects of Dibenamine and atropine on sweat secretions in the cat are studied by a quantitative procedure. Electrical stimulation of the lumbar sympathetic chain evokes transient electrical negativity in the skin of the footpad, relative to gland-free skin of thigh, back or ear.[4,5] The potential is the result of sweat gland activity, and its magnitude is a quantitative measure of the number of neuroglandular elements active. The response to a constant single shock to the sympathetic chain is relatively constant over long periods of time, permitting ready recognition of deviations due to injected drugs.

Methods and materials. In 5 cats, anesthetized with sodium pentobarbital, the lumbar sympathetic chain was exposed retroperitoneally. Single square wave shocks, 0.5 msec. in duration and of supramaximal intensity, were delivered to the L5-L6 interganglionic segment of the chain every minute for 5-15 minutes before, during, and for 30 minutes to one hour after drug infusion.

The potentials were recorded through Pb-$PbCl_2$ electrodes attached to the shaved skin of the thigh and to the ipsilateral hind footpad. The latter electrode, designed by pouring lead around a positive plaster impression of a cat's foot, established firm contact with all parts of the hairless foot and toe pads. The potentials were recorded without amplification on a string galvanometer or on a Hathaway galvanometer after amplification by a DC amplifier of the carrier type.*

The Dibenamine solution, prepared freshly in slightly acid saline, was administered through the femoral vein slowly (approximately 1 mg/min.) to avoid the complications of precipitous hypotension. Total dosage was recorded at each one-minute interval; final total dosages ranged from 15-20 mg per kilo of body weight. The pilomotor response of the tail to repetitive stimulation of the sympathetic chain was noted at the beginning and end of each experiment to assay qualitatively the effectiveness of the dosage.

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[1] Dale, H. H., and Feldberg, W., *J. Physiol.*, 1934, **82**, 121.

[2] Haimovici, H., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 40.

[3] Nickerson, M., and Goodman, L. S., *Fed. Proc.*, 1948, **7**, 397.

[4] Richter, C. P., and Whelan, F., *J. Neurophysiol.*, 1943, **6**, 191.

[5] Patton, H. D., *J. Neurophysiol.*, 1948, **11**, 217.

* The amplifier was designed and constructed by Mr. R. S. Bark, Department of Physiology and Biophysics, University of Washington School of Medicine.

Atropine sulfate was given in a similar fashion, except that the total dosage was administered rapidly.

Results. Slow intravenous infusion of Dibenamine in dosages (15 to 20 mg per kg) sufficient to block completely the pilomotor response of the tail, to produce miosis and to relax the nictitating membrane did not diminish the electrical response of sweat glands to preganglionic sympathetic stimulation. Fig. 1 shows the results of a typical experiment. At no time during the infusion did the magnitude of the potential drop below the control value. In some experiments longer post-infusion controls showed that the potentials were maintained at control heights as long as an hour after administration of the full dose. Indeed, in some animals the response increased moderately during and following Dibenamine dosage (Fig. 2). In only one experiment did the response show a significant decrease (35 minutes after full dosage); in this case it was found that the initially chosen stimulus strength was too weak, and the response was restored with increased strength of stimulus.

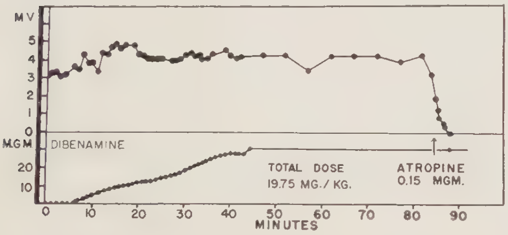

Fig. 1.

Upper graph, magnitude of footpad potential (m.v.) in response to supramaximal sympathetic stimulation. Lower graph, cumulative dose of Dibenamine. At the end of the experiment, injection of atropine rapidly abolished the responses.

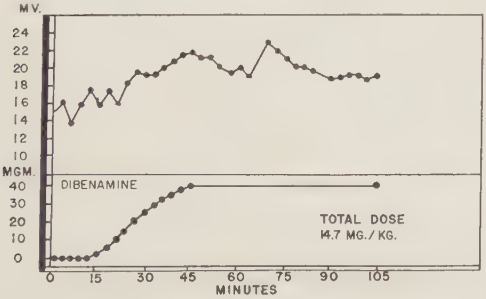

Fig. 2.

Increase in footpad potentials following Dibenamine administration.

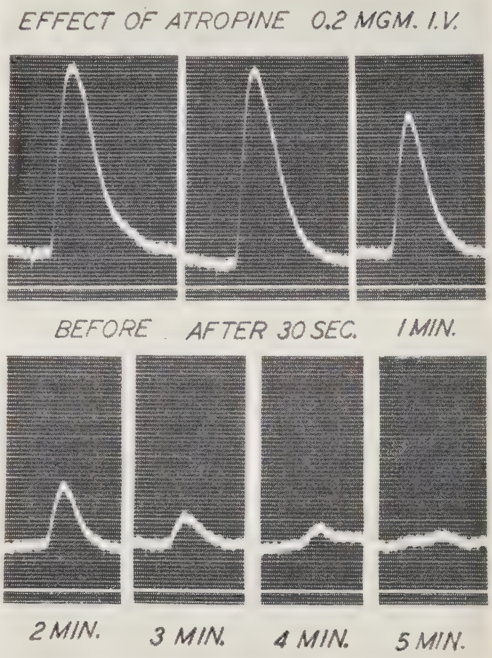

Fig. 3.

Potential responses of footpad to single shock stimulation of the sympathetic chain before and after injection of atropine.

Injection of atropine (0.1 mg per kg) produced a rapid and complete block of the sudomotor system. Fig. 3 shows the results of a typical experiment.

Discussion. The results indicate that sweat secretion in the cat is not blocked by Dibenamine, even with doses 3-4 times as great as that reported to block sweating in man. Miosis, relaxation of the nictitating membrane, and paralysis of the pilomotor system indicated that the dosage was adequate to block known adrenergic systems. In some animals the magnitude of the footpad potential actually increased during infusion of the drug.

This may have been due to vasodilation and increased blood supply to the glands.

On the other hand, irreversible sudomotor paralysis consistently followed injection of atropine. It is thus concluded, in agreement with Dale and Feldberg, that the sudomotor innervation in the cat is entirely cholinergic. Either there exists a marked species difference in cat and man in this respect, or the qualitative methods of observing sweating are misleading.

Summary. The effect of autonomic blocking agents on sweat gland response to single shock excitation of the sympathetic chain was studied in cats by a quantitative and objective method. Dibenamine, an adrenolytic and sympatholytic agent, failed to block sweat secretion whereas atropine consistently produced complete and irreversible sudomotor paralysis. It is concluded that the sudomotor innervation in cat is entirely cholinergic and possesses no adrenergic component, as reported in man.

16945

Stable, Reduced, Desiccated Streptolysin "O."*

ELIZABETH RANDALL AND LOWELL A. RANTZ.

From the Department of Medicine, Stanford University School of Medicine, San Francisco.

A technic has been described[1] for the determination of the antistreptolysin "O" titer of serum which involved the employment of a concentrated lysin, diluted and reduced to the active form with a solution of cysteine hydrochloride at the time of use. This method has proved to be very satisfactory. Large amounts of the lysin have been prepared not oftener than every 12 months and deterioration during storage at circa 2°C has rarely been observed. A stable material not requiring refrigeration nor reduction at the time of use would be most desirable. This report describes a procedure for the preparation of a desiccated, reduced streptolysin "O" which, upon reconstitution with distilled water, is immediately ready for use.

Streptolysin. Concentrated streptolysin "O", prepared exactly as described in the paper mentioned above, is used.[1] Complete removal of sulphate ions is determined after dialysis by the addition of a drop of 25 percent barium chloride solution to a small amount of the concentrated material. A precipitate forms after dialysis and the addition of sodium chloride. It should be removed by centrifugation.

Cysteine Hydrochloride. The dry salt is required.

Concentrated Buffer. A concentrated (4 times normal) buffered saline solution is prepared according to the following formula.

	g
NaCl	68.0
KH_2PO_4	45.2
$Na_2HPO_4 \cdot 12\ H_2O$	74.6
Distilled water to make 4 liters.	

After the salts have been dissolved the acidity is checked with a glass electrode pH meter and adjusted to final pH of 6.5. The solution need not be sterilized.

Buffered Solvent for Cysteine. Seven and two-tenths grams of solid NaOH is added to one liter of normal buffered saline (1 part of concentrated buffer saline diluted with 3 parts of distilled water). At the time of reduction and dehydration an M/5 solution of cysteine hydrochloride is prepared by dissolving 3.0 g of the dry salt in 100 cc of this alkaline buffer. Final pH should be 6.5 to 7.2. The solution need not be sterilized.

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* This investigation was conducted under the auspices of the Commission on Acute Respiratory Diseases, Army Epidemiological Board, Office of the Surgeon General, United States Army, Washington, D.C.

[1] Rantz, L. A., and Randall, E., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 22.

TABLE I.

	Volume of reagents			
	Lot 1		Lot 2	
	For 1 bottle, ml	For 100 bottles, ml	For 1 bottle, ml	For 100 bottles, ml
Vol. of concentrated lysin containing 40 combining units	.875	87.5	1.5	150.0
Vol. of M/5 cysteine	2.5	250.0	2.5	250.0
Vol. of normal buffer saline	(16.62)		(16.0)	
Vol. of conc. buffer saline	4.15	415.0	4.0	400.0
Total to be added to each bottle before lyophilization	7.52	7.52	8.0	8.0

Method. The object of the method is to have the lysin, salts and reducing agent combined in the proper proportions and in a volume conveniently small for lyophilization. Each finished bottle of dehydrated material contains exactly 40 combining units of streptolysin, the residue from 2.5 ml of M/5 cysteine solution and sufficient buffer salts so that the whole will be isotonic when reconstituted with 20 cc of distilled water. The following calculation is made: 20 ml less the volume of lysin containing 40 combining units plus 2.5 cc of M/5 cysteine equals the volume of normal buffer required. This value, divided by 4, gives the amount of concentrated buffer per bottle. The sum of the volumes of lysin containing 40 combining units, of M/5 cysteine, and of concentrated buffer saline is the amount of reduced buffered material to be transferred to each bottle before dehydration. The examples shown in Table I are illustrative.

The concentrated lysin, M/5 cysteine solution, and concentrated buffer are appropriately measured and mixed after complete preparations have been made for freezing and drying. The solution is dispensed with an accurate automatic pipette into standard 20 cc vials with an inside neck diameter of 12 mm. The maximum time which may elapse after reduction and before freezing is not known but 500 to 1,000 bottles may safely and satisfactorily be handled.

The filled bottles are vertically frozen at once in a shallow bath of methylcellosolve containing large amounts of dry ice, and desiccated from the frozen state in a commercial dehydrating apparatus at a pressure of 300 microns of mercury for 42 hours. The final moisture content is circa 2.5%. The vacuum is broken with sterile dry air and the bottles closed with a rubber flange stopper in a dry sterile stoppering cabinet and sealed with standard aluminum seals.[†]

The Test. One bottle of dehydrated, reduced lysin is reconstituted with 20 ml of distilled water. It dissolves at once to form a water clear solution and is ready for use. One combining unit is contained in 0.5 ml. The material may be used in any of the technics for determination of the antistreptolysin titer of serum. The dilution system previously described has proved to be very satisfactory.[1]

Stability. The dehydrated reduced lysin has been stored at 37°C, room temperature, and at 2°C for 18 months. Serial tests have been made and no loss of potency or alteration in the characteristics of the product has been noted. After reconstitution the material is highly unstable and should be used within 2 hours.

Discussion and Summary. A method is described for the preparation of a very stable, desiccated, reduced streptolysin "O". The material, when reconstituted with distilled water, is suitable for use in the determination of the antistreptolysin "O" titer of human serum. The availability of this material greatly simplifies this procedure since it is always immediately ready for use without the addition of the reducing agent. In addition, it may be shipped and stored conveniently since refrigeration is not required. Its production on a large scale or commercial basis would not prove difficult if a sufficient need for this material should arise.

[†] The cooperation of the Cutter Laboratories is gratefully acknowledged. Processing has been done in the plant of this company. Mr. B. E. Emery of that organization may be consulted for additional technical details.

16946

Influence of Heating on the Liberation of Certain Amino Acids from Whole Soybeans.*

H. C. HOU,[†] W. H. RIESEN, AND C. A. ELVEHJEM.

From Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

The effect of heating on the nutritive value of soybeans has been studied by a number of investigators. A review of such studies was made recently by Evans and McGinnis.[1] They further showed that the nutritive value of soybean proteins as determined by gain in weight per gram of protein consumed by chicks was increased when the soybean oil meal was autoclaved at 100°C, 110°C or 120°C for 30 minutes but to a smaller degree when it was autoclaved at 130°C for 30 or 60 minutes. The changes in nutritive value were found to parallel the availability of the methionine and cystine of the soybean.

Clandinin *et al.*[2] reported that heating solvent-extracted soybean flakes in an autoclave at 15 lb pressure for 4 minutes resulted in a meal of high nutritive value for the chick; however, when the heating was prolonged to 4 hours a decrease in the nutritive value was observed. These changes were shown by Riesen *et al.*[3] to be related to an increase in the amount of each of the essential amino acids liberated by pancreatic hydrolysis with the short period of heating and a decrease with the prolonged heat treatment.

More recently Klose *et al.*[4] reported that treatment of raw soybeans with steam at 15 lb pressure gave a product of maximum nutritional value for the rat after 10 to 15 minutes while heating beyond this time resulted in a gradual decrease owing to a resultant deficiency of methionine, lysine and leucine. Steam treatment at about atmospheric pressure for 30 minutes gave a product showing a slightly higher maximum and there was little decrease in nutritive value on prolonged heating.

Soybeans have been used in the Chinese diet for centuries and one of the methods of cooking is to boil in water for 2 to 3 hours until the beans are quite soft. The present investigation was designed to determine the effect of boiling soybeans in water at different periods of time on the liberation of certain essential amino acids by acid and by enzymatic digestion.

Methods. The soybeans used were recently harvested, dried seeds of the Hawkeye

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[†] On leave from the Institute of Nutrition, National Institute of Health, China, and on traveling fellowship of the World Health Organization of the United Nations and of The Williams-Waterman Fund for the Combat of Dietary Diseases.

[1] Evans, R. J., and McGinnis, J., *J. Nutrition*, 1946, **31**, 449.

[2] Clandinin, D. R., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *Poultry Sci.*, 1947, **24**, 150.

[3] Riesen, W. H., Clandinin, D. R., Elvehjem, C. A., and Cravens, W. W., *J. Biol. Chem.*, 1947, **167**, 143.

[4] Klose, A. A., Hill, B., and Fevold, H. L., *Food Tech.*, 1948, **2**, 201.

TABLE I.
Effect of Time of Boiling on the α-amino Nitrogen Liberation from Soybeans.

Time of boiling hr \ Time of digestion, hr	α-amino nitrogen (g %)			
	Acid digest	Pancreatic digest		
	10	0	6	25
0	4.51	.42	0.68	1.00
¼	—	.44	0.99	1.48
1	—	.54	1.63	2.00
2	4.56	.49	1.57	2.12
3	4.75	.52	1.60	2.10
4	4.70	—	—	—
12	4.52	.49	1.51	2.01

variety.‡ They were washed free from dirt wiped free from moisture and then air dried at room temperature. Weighed samples of whole beans were soaked in water for 3 hours and then boiled in water (about 5 times the weight of the beans), under reflux for varying periods of time. The soaked and the heated soybeans with liquid were then homogenized in a Waring blendor for about one-half hour at short intervals to prevent heating of the emulsion.

These emulsions were used for subsequent acid and alkaline hydrolysis by autoclaving at 15 lb with final volume at 20 ml of 3 N hydrochloric acid per 0.5 g soybean protein for 10 hours and 10 ml of 5 N sodium hydroxide per 0.5 g of soybean protein for 15 hours, respectively. Alkaline hydrolysis was carried out for the assay of tryptophan.

Enzymatic hydrolysis was carried out by shaking the emulsion with desiccated and defatted whole pancreas (VioBin 3 × USP Trypsin) in disodium phosphate buffer (pH 8.3) with toluene at 37°C for 0, 6 and 25 hours. The quantities used were 100 mg whole pancreas and 50 ml of 0.2 M Na_2HPO_4 to one gram of protein. Other details were similar to those described by Riesen *et al.*[3]

The liberation of α-amino nitrogen from the acid and pancreatic hydrolysates was determined by the nitrous acid micromethod of Van Slyke.

Amino acid assays were carried out according to the method of Henderson and Snell[5] except that in the present experiment *Leuconostoc mesenteroides* P60 was used for leucine and tryptophan while *Leuconostoc citrovorum* 8081 was used for arginine, cystine, histidine and methionine. Lilly's liver concentrate (Reticulogen 20 × USP) was added to the media for the latter organism at a level of 0.4 ml per 500 ml of medium (Steele *et al.*[6]).

Results and Discussion. Liberation of α-amino nitrogen. As shown in Table I the total α-amino nitrogen found in soybean after acid hydrolysis was unaffected by boiling for different periods of time up to 12 hours. With pancreatic digestion the amount of α-amino nitrogen liberated from raw soybean and that which had been boiled for 15 minutes was small but reached a maximum when the soybean was heated for one hour. Prolonged heating for periods of 2, 3 or 12 hours produced practically no difference in the total α-amino nitrogen liberated. These results are similar to those reported previously[3] for solvent-extracted flakes except that in the previous experiment heating at the higher temperature for 4 hours resulted in a decrease in α-amino nitrogen released by pancreatin. The fact that boiling for 15 minutes brought about a smaller release of α-amino nitrogen by pancreatin than boiling for longer periods is added evidence that the trypsin inhibitor was not the sole cause of the smaller release from raw soybean. This confirms the

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‡ Kindly supplied by Dr. J. H. Torrie of the Agronomy Department, University of Wisconsin.

[5] Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, 1948, **172**, 15.

[6] Steele, B. F., Sauberlich, H. E., Reynolds, M. S., and Baumann, C. A., *J. Biol. Chem.*, in press.

TABLE II.
Liberation of Amino Acids from Raw and Boiled Whole Soybeans.

	Amino acid N content of soybean (% of total N)*			% amino acids liberated by pancreatin (25 hr digestion)		
Boiling time, hr	0	3	12	0	3	12
α-amino nitrogen	67.3	71.0	67.5	22.2	44.2	44.5
Arginine	14.3	14.8	14.5	28.1	61.1	55.4
Cystine	0.50	0.58	0.57	30.2	76.6	60.6
Histidine	4.13	4.82	4.74	21.1	44.7	43.1
Leucine	4.41	4.22	3.99	30.0	45.7	50.7
Lysine	6.88	6.87	6.88	26.6	53.7	50.5
Methionine	0.60	0.62	0.63	33.5	72.3	63.9
Tryptophan	0.60	0.61	0.57	35.4	79.0	78.3
Total†	31.4	32.5	31.9	28.5	55.4	53.2

* Total nitrogen of soybean determined by Kjeldahl method was 6.69 g %.

† Amino acid N totals are sums of the percentage of the amino acids N liberated by acid or alkaline hydrolysis. Pancreatic hydrolysis totals are obtained by dividing the total amount of individual amino acids liberated enzymatically by the total liberated by acid or alkaline hydrolysis.

finding of Riesen *et al.*[3] and also Westfall and Hauge[7] although the latter workers believe that the trypsin inhibitor was the chief cause of poor utilization of the protein by the mouse.

Amino acid levels. The results of heating on the individual amino acid value of soybeans in the raw state and after boiling for 3 and 12 hours are shown in Table II. It will be noted that after acid hyrolysis the amino acid values for raw soybeans are essentially the same as those boiled for 3 or 12 hours. Riesen *et al.*[3] who used prolonged heating at 15 lb pressure found a decrease in the liberation of lysine, arginine and tryptophan. The difference may be due to the fact that the temperature in the experiment of Riesen *et al.* was much higher than that of the present experiment.

Recently Patton *et al.*[8] reported that boiling of soy globulin in 5% glucose with refluxing for 24 hours caused a considerable loss in 4 out of 10 essential amino acids determined, namely lysine, arginine, tryptophan and histidine, while boiling in water resulted in no change in these amino acids. Our results with whole soybean were similar to theirs with boiling in water and would indicate that the sugars and other carbohydrates in soybeans do not play a part in the loss of amino acids during boiling, although browning did occur in samples boiled longer than 3 hours. It appears that the loss of certain amino acids during boiling in the presence of glucose reported by Patton *et al.*[8] does not have a counterpart in the whole soybean moiety.

Block *et al.*[9] showed that baking of a cake mix of flour, sugar, egg white, lactalbumin, hydrogenated vegetable oil, dried yeast, molasses and salt with 25% of its calories as protein caused a decrease in the protein efficiency. The decrease they believe was possibly due to a partial destruction of lysine which occurred during the baking, or to a formation of a new peptide linkage by a reaction of the free carboxyl groups of the dicarboxylic amino acids with the α-amino groups of lysine, the new peptide linkage being resistant to enzymatic digestion but not to acid hydrolysis. Since during baking the temperature on the surface of the cake may be considerably higher than 100°C, a partial destruction of lysine may occur similarly to that shown by Riesen *et al.*[3] On the other hand the molasses may furnish a considerable amount of glucose to cause destruction of lysine as reported by Patton *et al.*[8]

Pancreatic digestion. The effect of boiling the soybean on the liberation of amino acids was quite evident after 25 hours of pancreatic digestion (Table II). The total α-amino nitrogen released from the sample boiled for 3 hours was double that of the raw sample. Prolonged boiling for 12 hours resulted in no further change. Similar release occurred with each of the 7 amino acids studied. Since previous work indicated that the 7 amino acids tested in this study might be susceptible to damage by heat and in view of the present findings no further study was made of the remaining essential amino acids.

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[7] Westfall, R. J., and Hauge, S. M., *J. Nutrition*, 1948, **35**, 379.

[8] Patton, A. R., Hill, E. G., and Foreman, E. M., *Science*, 1948, **108**, 659.

[9] Block, R. J., Cannon, P. R., Wissler, R. W., Steffee, C. H., Jr., Straube, R. L., Frazier, L. E., and Woolridge, R. L., *Arch. Biochem.*, 1946, **10**, 295.

The results of the present study do not support the view that methionine is released at a greater rate than leucine and lysine after heating (Melnick *et al.*[10]). It was shown here as well as in a previous report[3] that heat increases the release of all amino acids from soybean protein by pancreatin to a similar extent. It would appear that the sulfur amino acid deficiencies in raw soybean previously reported by Hayward *et al.*[11] are due to a limited content *per se* of these amino acids in the soybean protein. The low quantities of cystine, methionine and tryptophan in both raw and heated soybean are evident from Table II. Supplementation of the protein with cystine and/or methionine should increase the nutritional value of both raw and heated soybean. Furthermore since all amino acids are less available in raw soybean the critical nature of the sulfur amino acid deficiencies in this protein is even more striking than in the heated soybean.

It is of interest to point out that the percentage increase of total amino acids released by pancreatin over the percentage of α-amino nitrogen is considerably smaller than that reported previously.[3] This may be explained by the fact that the organisms used in the present experiment did not utilize peptides to such an extent as those organisms employed in earlier experiments.

The results of our study show that the method of cooking soybean used in China does increase the nutritive value of soybean by allowing a greater enzymatic release of essential amino acids and that there is no destruction on prolonged boiling up to 12 hours. In view of this and the fact that cooking at higher temperature (autoclaving at 15 lb) results in a deleterious effect on certain amino acids reported previously,[3] cooking of soybean in a pressure cooker should not be introduced until future studies have proven that under the ordinary cooking condition with a pressure cooker no harmful result is obtained.

Summary. A study was made on the effect of boiling on the liberation of amino acids by acid and enzymatic hydrolysis using microbiological assay methods.

Boiling up to 12 hours did not not affect the release by acid hydrolysis of α-amino nitrogen and of 7 essential amino acids studied.

Boiling for 15 minutes caused a slight increase in the release of α-amino nitrogen and amino acids by pancreatic digestion but boiling for 1 hour and longer up to 12 hours resulted in a marked increase. The increase in the enzymatic liberation of amino acids for periods of over one hour was about twice that liberated from raw soybean. Prolonged boiling up to 12 hours with definite browning did not result in a decrease in enzymatic liberation of amino acids from the "overcooked" soybean.

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[10] Melnick, D., Oser, B. L., and Weiss, S., *Science*, 1946, **103**, 326.

[11] Hayward, J. W., Steenbock, H., and Bohstedt, G., *J. Nutrition*, 1936, **11**, 219.

16947

Colorimetric Determination of Urethane as Ethyl Alcohol in Blood.*

NORWOOD K. SCHAFFER, FRANCIS N. LEBARON, AND BURNHAM S. WALKER.

From the Department of Biochemistry, Boston University School of Medicine.

Archer, Chapman, Rhoden and Warren[1] have described a method for the determination of urethane (ethyl carbamate) in blood in connection with urethane therapy in leukemia. Their method was based upon the hydrolysis of urethane to ethyl alcohol and determination of the latter by oxidation with dichromate and titration of excess dichromate by iodimetry. This procedure, in specimens of normal blood, gave a relatively high nonspecific "urethane" value of 3-5 mg %; the clinical levels were 10-20 mg %. No recovery experiments were reported.

For the purposes of studying the blood urethane level in patients receiving urethane locally for therapy[2] against gram-negative organisms in infected wounds, a method was needed with greater precision at lowel levels of urethane. Hemingway, Bernat, and Maschmeyer[3] have indicated the unsatisfactory results of iodimetry in micro-titrations of chromic acid, and have proposed the use of barium diphenylamine sulfonate as an indicator, and ferrous sulfate as the titrant. For convenience we have preferred a colorimetric procedure; for increased accuracy we have introduced a more concentrated blood filtrate. The ferrous titration has been, in our hands, equally precise but more time-consuming.

Reagents. 0.300% potassium dichromate. 27 N sulfuric acid. 3 vol. of concentrated acid added to 1 vol. of water. 50% sodium hydroxide (wt.-vol.). 2/3 N sulfuric acid. 10% sodium tungstate.

1:5 tungstic acid blood filtrate. 2 volumes of water are added to oxalated blood, preferably at least 12 ml for blood low in urethane, and the mixture is shaken occasionally for a few minutes. One volume each of 10% sodium tungstate and 2/3 N sulfuric acid is added. It is mixed well and allowed to stand for 5 minutes, after which it is centrifuged and the supernatant fluid is filtered.

Distillation apparatus. An all-glass distillation apparatus is used, consisting of a glass-stoppered 125 ml pyrex distilling flask with an air-cooled condenser ¼ inch in diameter and 15 inches long. This leads to a vertical delivery tube 7 inches long ending in an aeration bulb. The aeration bulb dips below the surface of the acid-dichromate solution contained in a 25 ml pyrex glass-stoppered graduated cylinder cooled in an ice water bath in a liter beaker. The ice bath rests on a wooden block high enough (about 3½ inches) so that the aeration bulb will be well above the 13 ml mark on the cylinder when the block is removed and the ice bath with the cylinder is lowered to the table.

Blank determination. 16 ml of tungstic acid filtrate and roughly 4 ml of water or less filtrate and enough water to make a 20 ml volume are added to the distilling flask containing a few glass beads. 1 ml of 0.300% potassium dichromate and 3 ml of 27 N sulfuric acid are added to a 25 ml glass-stoppered cylinder and mixed. The cylinder is placed in the ice bath with the ice water covering the 16 ml mark. The delivery tube is lowered into the cylinder so that the aeration bulb is near its bottom. The distillation is carried out with a micro-burner and must be fast enough to prevent the acid-dichromate solution from sucking back. About 8.5 ml are distilled. To stop the distillation, the ice bath with the cylinder is lowered to the table while the flask is still being heated. The outside of the delivery tube is washed with a few tenths ml of water, which is blown

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* Aided by a grant of the Committee on Therapeutic Research of the Council of Pharmacy and Chemistry of the American Medical Association.

[1] Archer, H. E., Chapman, L., Rhoden, E., and Warren, F. L., *Biochem. J.*, 1948, **42**, 58.

[2] Howe, C. W., *Surg., Gynec., and Obstet.*, 1948, **87**, 425.

[3] Hemingway, A., Bernat, L. A., and Maschmeyer, J., *J. Lab. and Clin. Med.*, 1948, **33**, 126.

into the cylinder by heating the flask. The volume in the cylinder is made to about 12.9 ml with additional distillate (or water).

The cylinder is stoppered, shaken, sealed with 27 N sulfuric acid, and heated at 85° for 60 minutes. It is cooled in water, made up to a volume of 13 ml and mixed. The contents are transferred to an Evelyn tube and read in the Evelyn colorimeter using a 440 mμ filter[4] after the instrument has been set to 100% transmittance with water. The preparation of a standard curve is described below. A similar standardization should be done by each user of the method.

Urethane plus blank determination. The 25 ml cylinder containing freshly prepared acid-dichromate solution is set up with the distillation apparatus as in the blank determination. Sixteen ml of tungstic acid filtrate or less filtrate and enough water roughly measured to make a 16 ml volume are added to the distilling flask. 4 ml of 50% sodium hydroxide are added, the flask is stoppered and the contents are mixed. A water bath is set up around the distilling flask and heated at boiling for 15 minutes. To stop this heating, first the flame under the boiling water bath is increased until the acid-dichromate solution is forced out of the delivery tube. Then the ice bath is lowered to the table, the boiling water bath is removed, the ice bath is replaced and the distillation is started immediately. The rest of the procedure is as in the blank determination. Since the dichromate color is very stable, the determination may be interrupted either before or after the 85° incubation by storage in the refrigerator. The urethane concentration is obtained by subtracting the concentration of the blank from that of the urethane plus blank.

Urethane standard curve and formula. Different amounts of urethane, varying from 0.15 to 1.3 mg, were analyzed by the above method. Each quantity was determined 4 to 10 times. The average values of the optical densities were plotted against mg urethane (Fig. 1). The relationship was linear in this range. The value of the optical density with no urethane was taken as that of a determination with 16 ml water plus 4 ml of 50% sodium hydroxide. This value, 0.4297 ± 0.0068, was slightly lower than that, 0.4436 ± 0.0056, of the acid-dichromate solution simply diluted to 13 ml. The slope of the line was taken as the average of the separate slopes, omitting that of the 1.3 mg urethane point. Amounts of urethane greater than 1.2 mg showed a consistent deviation from this line. The formula of this line is:

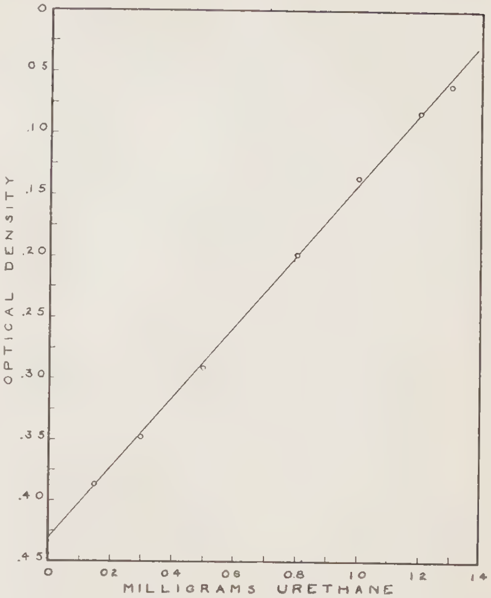

FIG. 1.
Urethane standard curve.

$$\text{Mg Urethane} = 3.47\ (0.4297 - L).$$

where the absorbency of the sample, or "optical density", $L = \log 100 - \log G$, and G is the Evelyn galvanometer reading (percentage transmittancy).

Precision of method. The precision of the above urethane standard determinations is given in Table I. Since the error of a single determination is within ± 2 standard errors, at 0.15 mg urethane the error is about 10% and this decreases to about 2% with 0.5 to 1.2 urethane.

Recovery of urethane added to blood. The recoveries of urethane added to whole blood at concentrations from 3.9 to 43.5 mg % using a 1:5 tungstic acid filtrate are recorded in Table II. These vary from 99 to 111%.

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[4] Gibson, J. G., and Blotner, H., *J. Biol. Chem.*, 1938, **126**, 551.

TABLE I.
Precision of Urethane Standard Determinations.

Urethane, mg	0.15	0.3	0.5	0.8	1.0	1.2
Standard error, in %	5.1	2.4	0.7	1.2	0.5	0.7

TABLE II.
Recovery of Urethane Added to Blood.

Urethane added, mg %	Urethane found, mg %	% recovery
3.9	4.2	108
5.7	6.3	111
7.8	7.7	99
11.1	12.3	111
15.9	16.1	101
18.5	19.3	104
19.2	19.0	99
32.3	34.5	107
43.5	45.7	105

Such bloods gave the same urethane values when they were previously hemolyzed with one tenth volume of 1% saponin. The 1:10 tungstic acid filtrate was abandoned because the recoveries were more variable at low urethane concentrations.

Specificity of method. In 5 normal bloods we have found non-specific "urethane" values varying from 0.1 to 1.4 mg % and averaging 0.8 mg %. Five other bloods from patients receiving drugs (sulfadiazine, streptomycin, penicillin, phenobarbital and amytal) in therapeutic doses gave "urethane" values from 0 to 1.1 mg % and averaging 0.5 mg %. These "urethane" values are zero within the accuracy of the method, hence urethane values below 1.5 mg % have no significance.

Clinical blood urethane levels. Patients with wound infections receive urethane locally.[2] In 2 such cases, we have found the blood urethane to be 3.1 and 5.6 mg %, after 4 and 8 days of treatment, respectively.

Application of method for ethyl alcohol determination. The blank determination in the urethane method may be used for the determination of ethyl alcohol since the conditions of the distillation and incubation were chosen to give quantitative recoveries of ethyl alcohol in amounts yielded in the urethane determination, that is, about ½ the amount of urethane. A standard alcohol curve was constructed from dilutions of a standard alcohol solution prepared from a weighed quantity of absolute ethyl alcohol. The curve was linear up to 0.4 mg alcohol, the 0.6 mg point deviating about 5 percent from the line. The equation of the line up to the 0.4 mg point was:

Mg Ethyl Alcohol = 1.65 (0.4436 — L). Strictly speaking the optical density at zero alcohol (0.4436 in the equation) should be that of a blank distillation with water.

Summary. A colorimetric method is described for the determination of urethane in blood, which gives 99 to 111% recoveries in the range 4 to 44 mg % urethane, and a non-specific "urethane" value of normal blood of 0.1-1.4 mg %.

A part of the method may be directly used for the determination of ethyl alcohol.

16948

Agglutination of Sea Urchin Eggs and Sperm by Basic Proteins.

CHARLES B. METZ. (Introduced by J. S. Nicholas.)

From Osborn Zoological Laboratory, Yale University, New Haven, Conn.

It is well known that basic proteins (protamines and histones) react with a wide variety of unrelated substances forming precipitates (with proteins, nucleic acids, etc.), causing cell agglutination (*i.e.* erythrocytes,[1] bacteria[1]) and exerting various physiological effects (*i.e.* parthenogenesis,[2] bacteriostasis[3]).

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[1] Lajmanovich, S., and Mittelman, N., *Rev. Inst. Bact.*, "Carlos G. Malbran," 1944, **12**, 320.

It is not surprising, therefore, that such substances precipitate the jelly coat of sea urchin eggs, agglutinate sea urchin eggs, and agglutinate the spermatozoa of a variety of forms.[4,5] It is unfortunate, however, that another sea urchin egg jelly precipitin and egg agglutinin, namely antifertilizin, should be confused with basic proteins from the sperm nucleus.

In a recent study Hultin[6] attempts to show that the sea urchin egg jelly precipitins (egg agglutinins) extracted from sea urchin sperm by Frank[7] (by heating sperm) and Tyler[8,9] (by freeze-thawing or extraction at pH3) are basic proteins, that they are non-specific in their action and that they are obtained from the sperm nucleus. Hultin maintains that the extracts of Frank and Tyler do not contain the sperm surface substance, antifertilizin, that reacts with the specific sperm agglutinin, fertilizin, obtained from eggs. The last is a particularly important charge since much of our present knowledge of the role of specific substances in fertilization stems from studies on sperm extracts as prepared by Frank and Tyler.

Hultin's claims are based on the following observations: (1) basic proteins obtained by extracting sperm with acid (pH 0.9 to 1.0) agglutinated eggs of various echinoid species indiscriminately, (2) basic proteins split from sperm nucleoproteins (cytoplasm free) caused egg agglutination and jelly precipitation, (3) sperm cytoplasm prepared by citric acid extraction had no effect on eggs or egg jelly of the homologous species (*Arbacia lixula*) whereas acid extracts of the residual sperm nuclei gave strong positive reactions with eggs, (4) egg agglutinin and jelly precipitin samples prepared from sea urchin sperm by heating or extraction with weak acid (pH 3) gave positive tests for desoxyribosenucleic acid. Hultin makes no statement regarding the presence or absence of free basic protein in these last preparations.

Unfortunately Hultin reports no test for cross agglutination of sperm by heterologous fertilizin or cross agglutination of eggs by the sperm extracts of Frank and Tyler. Without such data as a standard of specificity his studies on specificities of sperm extracts have little significance since cross reactions between echinoid sperm and fertilizin are known. Hultin mentions no controls testing the effect of his basic protein extraction procedure (extraction at pH 1) or the effects of the cytoplasm extraction procedure (citric acid extraction) on antifertilizin samples as prepared by Frank and Tyler. To claim identity of the egg agglutinins of Frank and Tyler with the basic protein fraction it would appear essential to show that the former retains its activity when subjected to the extraction procedure required for preparation of the basic protein.

Some years ago the writer[4] began a study of the effects of basic proteins on eggs and sperm. It was found that basic protein prepared by acid extraction (pH 0.5 to 1.0) of sperm of 3 sea urchins (*Strongylocentrotus purpuratus, Lytechinus anemesis, Arbacia punctulata*) not only agglutinated homologous eggs, but agglutinated homologous sperm as well. The latter property is definitely not shared with sea urchin antifertilizin as prepared by Frank and Tyler. Such basic protein preparations also agglutinated sperm from a variety of unrelated species. Thus *Arbacia* basic protein agglutinated sperm of seven (three molluscs, four echinoderms) out of ten forms; *S. purpuratus* basic protein agglutinated sperm of 5 echinoderms and one mollusc. These and a variety of other basic protein sperm agglutinations will be reported in detail elsewhere.

Basic proteins give strong non-specific egg jelly precipitation and egg agglutination reactions as Hultin states. Thus basic protein

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[2] Loeb, J., Artificial Parthenogenesis and Fertilization, Univ. Chicago Press, 1913.

[3] Negroni, P., and Fischer, I., *Rev. Soc. Argentina Biol.*, 1944, **20**, 487.

[4] Metz, C. B., Doctorate Thesis, California Institute of Technology, 1942.

[5] Hultin, T., *Ark. Kemi, Mineral., Geol.*, 1947, **24B**, 12.

[6] Hultin, T., *Pubbl. staz. zool. Napoli*, 1947, **21**, 2.

[7] Frank, J. A., *Biol. Bull.*, 1939, **76**, 190.

[8] Tyler, A., *Proc. Natl. Acad. Sci. U. S.*, 1939, **25**, 317.

[9] Tyler, A., and O'Melveny, K., *Biol. Bull.*, 1941, **81**, 364.

preparations from sperm of 7 forms (one annelid, 3 molluscs, 3 echinoderms) all agglutinated *Arbacia eggs*. It is of interest also that seminal fluid from the 3 molluscs agglutinated the *Arbacia* eggs. However, the basic protein egg agglutinins prepared from sea urchin sperm appear to be separate and distinct from the egg agglutinin (antifertilizin) prepared by freeze-thawing sperm. This follows from the fact that the egg agglutinating property of such antifertilizin was reduced (*S. purpuratus*) or disappeared entirely (*Arbacia punctulata*) when the antifertilizin preparations were subjected to the procedure used to extract basic protein. Thus, a sample of *Arbacia* antifertilizin (titer 16), prepared by freeze-thawing sperm, was adjusted to pH 0.8. A precipitate which formed at pH 2 to 3 was centrifuged off 4 hours later and the supernatant was neutralized. The sample was diluted to only one half the original concentration by addition of acid and base. It had no effect on the jelly coat of *Arbacia* eggs. It appears then that the antifertilizin was either inactivated by the acid or that it separated out in the precipitate (not tested further). Extracts prepared from whole sperm by this method gave powerful egg and sperm agglutination. Further evidence that the egg agglutinins in sperm extracts prepared by pH 1 extraction (basic proteins) and by freeze-thawing (antifertilizin) are separate and distinct was obtained in a neutralization experiment. A pH 1 sperm (*S. purpuratus*) extract which agglutinated both eggs and sperm was mixed with an active antifertilizin extract prepared by freeze-thawing sperm. The resulting mixture had no effect upon either eggs or sperm. Apparently the egg agglutinins in the two extracts can not coexist.

One may conclude from these experiments that at least 2 distinct jelly precipitating and egg agglutinating agents can be extracted from sea urchin sperm. The egg agglutinin(s) in pH 1 sperm extracts is probably a basic protein which acts in a highly non-specific fashion. The freeze-thaw and probably also the heat and pH 3 sperm extracts contain a different egg agglutinin(s), namely antifertilizin. For a discussion of antifertilizin specificity the reader is referred to Tyler's[10] recent review. The electrophoretic studies of Runnstrom, Tiselius and Vasseur[11] and Tyler[10] indicate that antifertilizin preparations contain a single acidic protein. In view of the foregoing the writer believes that Hultin has confused these two egg agglutinating substances obtainable from sperm. In the cases where basic protein extracts agglutinate homologous sperm as well as homologous eggs, this confusion can be rather readily avoided since antifertilizin preparations act on eggs but not on homologous sperm.

Summary. The agglutination of sea urchin eggs and sperm by basic protein extracts of homologous and unrelated sperm (reported by Hultin,[5,6]) is confirmed. Experiments are cited which show that antifertilizin, another egg agglutinin obtainable from sperm, is not present in or identical with the egg agglutinating basic protein fraction of sea urchin sperm as Hultin claims. Thus two distinct egg agglutinins are obtainable from whole sperm. One of these, antifertilizin, is obtained by heating, freeze-thawing or pH 3 extraction (Frank,[7] Tyler,[8,10]); the other, a basic protein(s), is obtained by pH 1 extraction of sperm.

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[10] Tyler, A., *Phys. Rev.*, 1948, **28**, 180.

[11] Runnstrom, J., Tiselius, A., and Vasseur, E., *Ark. Kemi, Mineral., Geol.*, 1942, **15A**, 16.

16949

Effect of Glycine Upon Action of Insulin in Rabbits.

J. M. Johlin. (With the technical assistance of Patricia W. Proctor.)

From Department of Biochemistry, Vanderbilt University, School of Medicine, Nashville, Tenn.

It was found by Pollak[1] that a pronounced hyperglycemia is produced in rabbits by the subcutaneous injection of a 5% solution of glycine. One gram of glycine was found to raise the blood sugar level of an animal weighing 1700 g from 90 to 160 mg % within 2 hours. Izumi[2] and Schenk[3] made further studies of the effect of amino acids upon the blood sugar level of rabbits. Izumi found that glycine administered intravenously (1.7 g per kg body weight) caused but a slight rise in the level of blood sugar, producing no subsequent lowering below the normal level, and that glycine administered perorally (2.675 g per kg) caused a slight lowering in the course of a 4 hour period. He pronounced the general effect of administering protein hydrolysates to be that of mobilizing blood glucose either from glycogen stores or by its formation from amino acids. Schenk[3] made a comprehensive study of the effect which the peroral administration of relatively large amounts of amino acids and of mixtures of these has upon the blood sugar level. Of those amino acids which were found to lower this level the effect of glycine was the most pronounced. In determining the amount of glycine to be administered the weight of the animal was not taken into account. The response was, however, interpreted in accordance with the amount administered. One gram was found to produce a *hyper*glycemia while the generally administered dose of 1.5 g was found to produce a *hypo*glycemia of varied degrees of intensity in different animals. Thus, the hypoglycemia produced by 1.5 g in an animal weighing 2810 g was more pronounced than that in one weighing but 1260 g. The hypoglycemic state disappeared within 8 hours after the administration of the glycine. Self-administered doses were found to produce a similar hypoglycemia in man.

When relatively small amounts of insulin were injected subcutaneously immediately after the feeding of glycine the decrease in the level of blood sugar was found to be the summation of that produced independently by the insulin and by the glycine.

It was found in this laboratory, using rabbits which had been carefully standardized with respect to their insulin tolerance, that small amounts of glycine (3 mg per kg) which were injected simultaneously with insulin, either subcutaneously or intravenously, did not significantly affect the insulin tolerance of these animals immediately. When the same rabbits were retested some weeks later, however, the effect which the administration of glycine had produced was found to be so pronounced as to cause these animals to reach the convulsive state when they were injected with but one-half of the originally determined convulsive dose of insulin and to promote a corresponding decrease in the level of the blood sugar. Thus, three rabbits which had received from 3 to 5 intravenous injections of insulin and glycine on alternate days late in February did not exhibit any significant response due to the glycine at that time. The same rabbits, upon receiving one or two subcutaneous injections of a mixture of glycine and insulin late in May, reached a convulsive state when the amount of insulin administered was but one-half that of the originally determined convulsive dose, (0.6, 0.7, and 0.7 units per kg respectively). These amounts constituting but one-half of the original convulsive dose are smaller than any which had heretofore been found to be required in bringing any normal rabbit to a convulsive state. Two other rabbits, upon receiving subcutaneous injections of glycine

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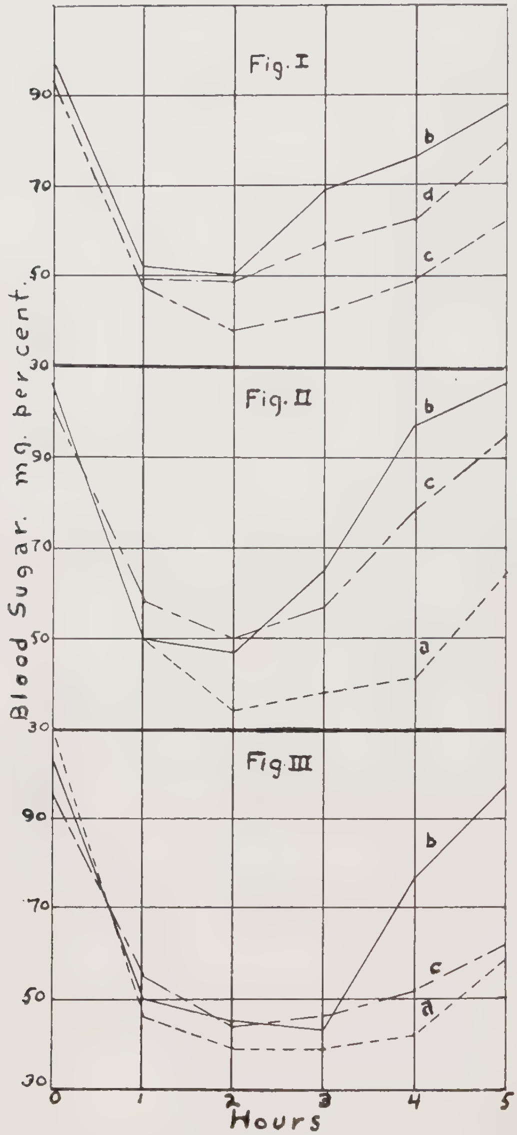
[1] Pollak, L., *Biochem. Z.*, 1922, **127**, 120.

[2] Izumi, K., *Biochem. Z.*, 1932, **248**, 383.

[3] Schenk, E. G., *Arch. Exp. Pathol. and Pharmacol.*, 1932, **167**, 201.



FIGS. 1-3.

Curve *a* of Figs. 2 and 3 shows the effect of a subconvulsive dose of insulin upon the blood sugar level of rabbits; curve *b* of each figure that of one-half the convulsive dose of insulin *before* treatment with glycine; curve *c* of each figure that of one-half of the convulsive dose of insulin *after* treatment with glycine; curve *d* of Fig. 1 that of one-half of a convulsive dose of insulin at a later period after treatment with glycine.

and insulin late in March, did not exhibit any significantly abnormal insulin response due to the glycine at that time. When the same rabbits received subcutaneous injections of insulin, with and without glycine, late in May, they were found to reach a convulsive state upon receiving but one-half of the originally determined convulsive dose of insulin.

This effect of glycine upon insulin action is more fully demonstrated by the following more recent data which were obtained in more carefully controlled experiments. These data, which are illustrated by the curves of Fig. 1 to 3, indicate the progressive change in the blood sugar level of rabbits for a 5 hour period following the subcutaneous injection of insulin, of glycine, and of a mixture of these. In all instances where glycine was administered the dose consisted of 3 mg per kg body weight. Five per cent solutions of glycine were used.

The data represented in Fig. 1 were obtained with a group of 4 rabbits (male albinos) which, as in all other experiments, had been carefully standardized with respect to their insulin tolerance. The data of curve *b* were obtained on Nov. 10th after the injection of one-half of the convulsive dose of insulin. Each animal was then injected with a mixture consisting of one-half of its convulsive dose of insulin and the usual amount of glycine on each of the following dates: Nov. 12th, 15th, 18th, 20th. The average blood sugar data obtained on each of these dates showed no significant deviation from those indicated by curve *b*. On Nov. 27th and 30th each animal again received the same injection of insulin and glycine as on Nov. 12th through 20th. The average data of these 2 days are given by curve *c*. They indicate a pronounced lowering of the blood sugar level during this 5 hour period as a consequence of the previous treatment with glycine. On Jan. 17th these rabbits were again injected with the same dose as on Nov. 27th. The data obtained, which are those of curve *d*, indicate a sustained effect due to the earlier injections of glycine.

The data of Fig. 2 were obtained with a second group of 4 rabbits which received similar treatment to those represented by Fig. 1. Injections of a mixture of glycine and one-half of the convulsive dose of insulin were given subcutaneously on Jan. 4th, 6th, 8th, and 10th. A similar injection was given each animal on Jan. 18th. Two of these (male albinos) reached the convulsive state after receiving the injection of Jan. 18th and their data are not included among those of Fig. 2. The other 2 rabbits (male grey) appeared to be somewhat less susceptible to the treatment than were the 2 albinos. Their data are represented by Fig. 2. The data of curve *a* were obtained on Dec. 20th following the injection of a dose of insulin slightly less than the convulsive dose and those of curve *b* on Dec. 22nd following the injection of one-half of the convulsive dose of insulin. Those of curve *c* were obtained on Jan. 18th following the injection of glycine and one-half of the convulsive dose of insulin. A definite effect of glycine on insulin action is also indicated here.

The data of Fig. 3 were obtained with a third group of 2 rabbits (black, male). After determining the response to a subconvulsive dose of insulin (curve *a*) and to one-half of a convulsive dose (curve *b*) these animals were injected with glycine only on Jan. 6th, 7th, 8th, 10th, and 12th. On Jan. 18th they received one-half of the convulsive dose of insulin but no glycine. A comparison of curve *c* with curves *a* and *b* indicates the effectiveness of the initial treatment with glycine upon the subsequent action of the injected insulin.

Many variables suggest themselves for further investigation. In view of the concern over the prediction of a possible shortage of insulin in the near future these experimental facts would seem of interest to a further search for a more favorable insulin economy. It should also be of interest to note how a similar treatment might affect humans who are predisposed to the conditions of diabetes.

Summary. Repeated injections of small amounts of glycine (3 mg per kg body weight) appear to increase the action of subsequent injections of insulin for a prolonged period of time.

The writer wishes to thank the donors of the Walter C. Hadley Fund for the generous aid which helped make this investigation possible.

16950

Production of Irritative and Destructive Changes in the Gastric Mucosa Followed by Regeneration.*

Enrique Sanchez-Palomera and Owen H. Wangensteen.
From the Department of Surgery, University of Minnesota Medical School, Minneapolis.

Some progress has been made in the last 3 decades concerning our knowledge of the normal and pathological physiology of the stomach. Special emphasis has been lent study of the functions of the peptic and parietal cells; by contrast, our knowledge of the gastric mucous epithelium and its function is meagre. Hollander and his associates have been probing with care for some time this aspect of the problem of functions and morphology of the gastric mucosa; with few exceptions the functions of the gastric mucous epithelium and the other mucous secreting cells have not been given due consideration.

In this study, investigations of some functions of the mucous secreting cells were carried out; also the mechanism of regeneration of the gastric mucosa was studied.

Methods and experiments. The gastric mucous secreting cells were stimulated by placing various substances into the stomach with both ends ligated. Cats and dogs were employed in the experiment. Mustard oil and clove oil in concentrations of from 0.5 to 2 % (in corn oil), and eugenol in watery solutions up to 10% were used.

The animals were anesthetized with nembu-

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* These studies were supported by a grant from the Committee on Food Research, Quartermaster Food and Container Institute for the Armed Forces, Chicago, Ill. Contract No. W11-009-q.m.-70215.

tal in doses of 15 mg per pound. When the operation was completed the animals were allowed to recover. After periods varying from 3 to 10 hours following operation, the animals were sacrificed.

In another small group, after the period of exposure to the irritant, the abdomens of the animals were re-opened, the ligatures on the stomach were removed, the stomach was emptied and the wound was closed. The same procedure was repeated 2 or 3 weeks later. In all cases biopsies were taken before and after the completion of the experiment. In the group of surviving animals, biopsies were also taken 24 to 48 hours after the stimulation was stopped.

The specimens were immediately fixed with formalin and stained with hematoxylin-eosin and Mayer's muci-carmin.

Comparative studies of the action of the irritants on closed loops of small bowel and colon were also made. In these cases the procedure was essentially the same as that described by Florey and Webb.[1]

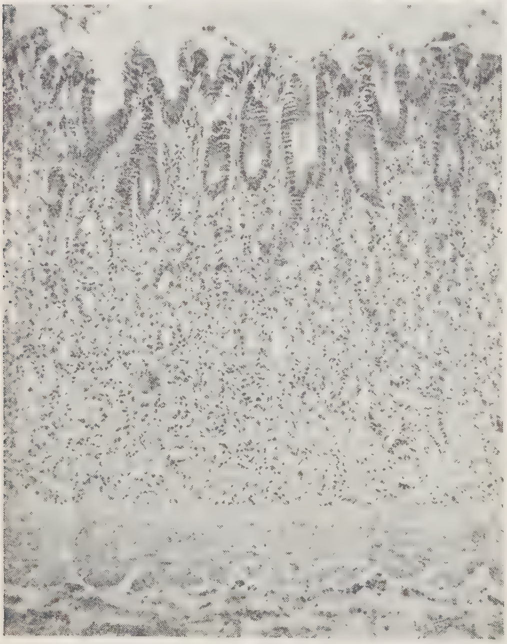

FIG. 1.
Stomach of a cat after 7 hours exposure to 1⅓% solution of mustard oil in corn oil. Early stage of mucosal changes; deepening of the foveolae, early epithelial desquamation. (Mayer's muci-carmin stain in all the microphotographs.)

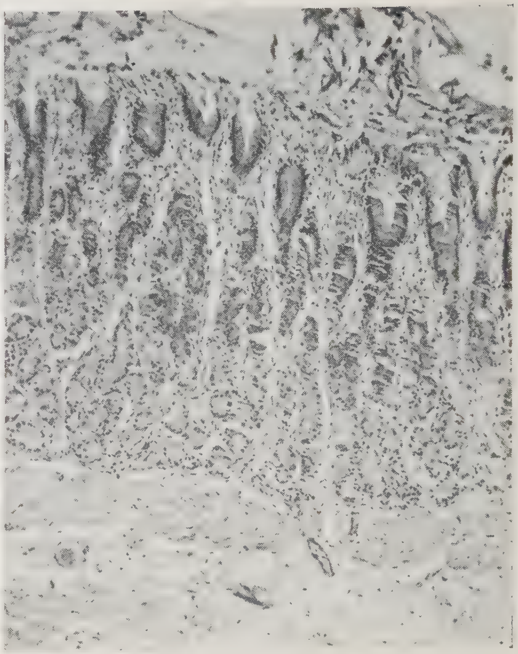

FIG. 2.
Stomach of a dog after 6 hours exposure to 10% aqueous solution of eugenol. This shows a more advanced stage than is shown in Fig. 1. There is definite loss of epithelium in the inter-foveolar areas.

Result. The results obtained in the colon and small bowel by Florey and Webb[1] were confirmed. In short, depletion of the goblet cells of the colon was readily produced. Similarly, diminution of the number of goblet cells was obtained in the small bowel.

In the stomach profuse macroscopic secretion of mucus was obtained with no obvious histological change. When the stimulation was stronger or the period of application increased, necrosis of the mucosa occurred. The process of necrosis was found to develop in the same general manner in colon, small bowel and stomach, except for the fact that the small bowel was the most susceptible and the stomach quite resistant to the irritative action. Edema, hyperemia, extravasation of red blood cells and leucocytic infiltration were noticed first. Later on, the most superficial portions

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[1] Florey, H., and Webb, R. A., *Brit. J. Exp. Path.*, 1931, **12**, 286.

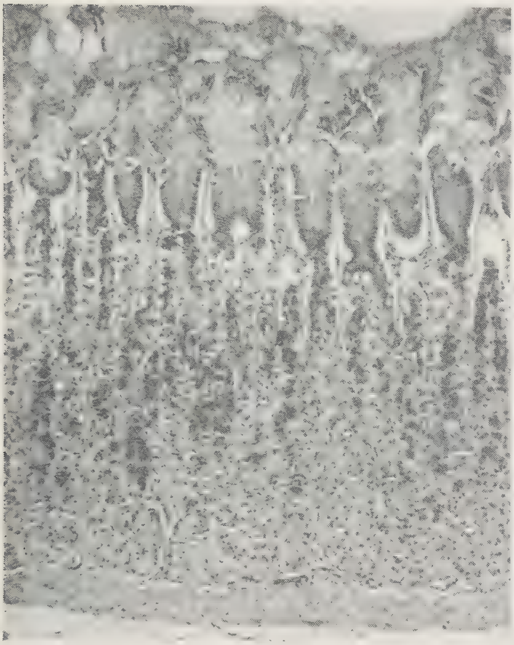

Fig. 3.
Stomach of a cat after exposure to 10% eugenol solution for 12 hours. Careful fixation of the whole stomach and attached mucus. The section shows a layer of epithelium already shed sticking to the new one.

of the epithelium were destroyed; the exposed cells of the stroma disintegrated and became spread over the denuded surface. A section, at that stage, showed an "umbrella" formed by cellular debris and mucous covering the mucosa. This layer acted as a protective covering and prevented necrosis of the walls of the pits. Further action of the irritant caused a progressive destruction of the remaining mucosa until ulceration occurred.

In the stomach, necrosis and regeneration were more thoroughly studied when irritants were applied to the mucosa. Deepening of the foveolae was first observed. (Fig. 1) Some time later the most superficial segments of epithelium became detached from the body of the mucosa, leaving only the deepest portions of the foveolae. (Fig. 2) If irritation ceased at this stage immediate regeneration took place. Under a protective layer of cellular debris and mucus, (Fig. 3) similar to the crust forming after injury to the epidermis), proliferation started at the bottom of the gastric pits. Growth of the cells from these areas restored the continuity of the surface epithelium and the mucosa looked thinner thereafter. The healed erosions in human stomachs show similar mucosal thinning. The process may be compared with the regeneration of the epithelium around skin appendages and the tips of the dermal papillae. If the entire thickness is destroyed, regeneration takes place from the margins of the wound. Similarly, if the entire thickness of the gastric mucosa is destroyed, regeneration takes place from the margins of the wound. Ferguson[2] demonstrated this experimentally and it can be seen also in pathological human material.

If destruction had not reached the bottom of the pits, it was followed by complete regeneration of the epithelial continuity in less than 48 hours. (Fig. 4)

In the cases in which a period of acute irritation was followed by recuperation of the animal, the procedure was repeated subse-

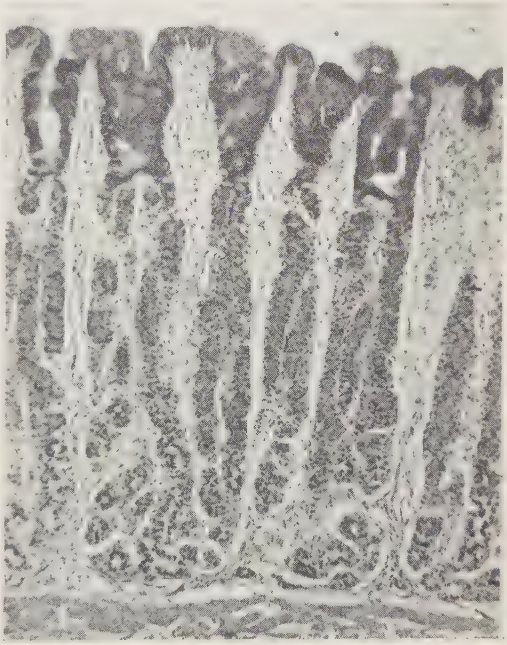

Fig. 4.
Same stomach as in Fig. 2, after regeneration. This biopsy was taken 40 hours after the stimulation was discontinued. The dark stained cells reaching almost to the muscularis mucosa are mucus-neck cells.

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[2] Ferguson, A. N., *Am. J. Anat.*, 1928, **42**, 403.

quently. Biopsies of the mucosa before this second period of irritation was started showed changes consisting of cystic areas scattered in the body of the mucosa, areas of degeneration and a ragged contour of the epithelial surface as observed in the section; moreover, some foveolae were deeper than others, instead of exhibiting as is usual a fairly even depth. The mucosa, at this stage, proved to be more susceptible to the action of the irritant than in the initial period.

Discussion. The regeneration of the gastric mucosa as described may explain, in a very simple way, the much discussed resistance of this viscus to auto-digestion. We know that mucous secretion affords a protective mechanism to the gastric mucosa. The surface mucus restrains the acid-peptic juice from coming into direct and intimate contact with the mucosa. Evidence of this is offered by the experiments described, and has been obtained among others by Whitlow.[3] It is also known that mucus is increased whenever any kind of trauma, either mechanical or chemical, is applied to the mucosa. If, in addition to the layer of surface mucus, one considers the mucus contained within the epithelial cells, the concept of a more effective barrier suggests itself. Cells and mucus, the latter tenaciously adherent to the former, offer a protection which can be favorably compared to that present in the other segments of the gastro-intestinal tract. Moreover, before the cells of the epithelium loose their ability to secrete mucus they are shed and are immediately replaced by a layer of new ones, with a full secretory capacity. (Fig. 4)

If the rate of dissolution of the mucosa exceeds the rate of regeneration which may be variable, mucosal defects form, from which ulcers of the wall may develop. In the main, the regenerative capacity of the gastric mucosa is great and this condition accounts reasonably for the circumstance that the gastric mucosa does not digest itself.

Dragstedt and Vaughn[4] observed that spleen and kidney transplanted to the stomach were covered by a layer of columnar epithelium. Similarly, Varco and Wangensteen[5] noticed that omentum employed to cover stomach defects (in which approximation of the gastric walls was precluded by the insertion of metallic rings), became covered by gastric epithelium. Protection against digestion in these tissues was very likely afforded by the newly-formed epithelial layer. This protective mechanism, the "mucous barrier," as it has been called by Farrell,[6] Ivy,[7] and Hollander,[8] may be of greater importance in the pathogenesis of gastric ulcer than previously considered.

Conclusions. 1. Changes in the gastric mucosa produced by continuous irritation are described.

2. The sequence of events following irritation is compared in colon, small bowel, and stomach.

3. The process of regeneration after superficial destruction of the mucosa is described and is offered as a possible simple explanation for the resistance of the stomach to auto-digestion.

4. The possible implication of this mechanism in the pathogenesis of gastric ulceration is suggested.

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[3] Whitlow, J. E., Thesis, M.S., 1920, Loyola Univ. Med. School.

[4] Dragstedt, L. R., and Vaughn, A. M., *Arch. Surg.*, 1924, **8**, 791.

[5] Varco, R. L., and Wangensteen, O. H., unpublished observations, 1940.

[6] Farrell, J. T., *Am. J. Phys.*, 1928, **85**, 672.

[7] Ivy, A. C., *J. Nat. Cancer Inst.*, 1945, **5**, 313.

[8] Hollander, F., Stein, J., and Lauber, F. U., *Gastroent.*, 1946, **6**, 576.

16951

Effect of DDT on Functional Development of Larvae of *Rana pipiens* and *Fundulus heteroclitus.*

EVELYN SCHREIMAN[†] AND ROBERTS RUGH.[*]

From the Biology Department, Washington Square College.

The effect of DDT (1-trichlor-2, 2-bis-p-chlorophenyl ethane) on adult fish, frogs,[1] and mammals,[2] has been reported recently. The general results indicate that DDT specifically affects the nervous system, a fact previously demonstrated in studies on insects. It seemed desirable to determine the stage in early embryonic development when DDT first affects the central nervous system. For this study poikilothermous and aquatic forms were chosen, namely the embryos of the frog *Rana pipiens* and the fish *Fundulus heteroclitus.*

Materials and methods. A total of 400 larvae of *Rana pipiens* and 250 of *Fundulus heteroclitus* were used in this study. The larvae were introduced into the experimental medium at various stages of development, for varying periods. The stages of *Rana pipiens* are those established by Shumway[3] and for *Fundulus heteroclitus* by Oppenheimer.[4]

The experimental medium consisted of a saturated solution of DDT in spring water for Rana and in distilled water for Fundulus (the latter being able to survive well in distilled water). DDT is quite insoluble but it is estimated that when 4 parts per million are used, that at least 1 part per million goes into solution.

Rana pipiens larvae were provided with 50 cc of medium for 25 larvae in a 250 cc finger bowl while *Fundulus heteroclitus* larvae were placed 50 to a finger bowl of 75 cc of medium. The experimental media were changed daily to keep the concentration at the experimental level and to avoid chemical deterioration. The controls were treated in an identical manner except for the use of DDT. Laboratory temperatures of 23°C to 25°C were used for all larvae.

The response to stimulation was measured by the Detwiler[5] "race-track" method. The photographs were taken with a Mifilmca Adapter, through the low magnification microscope, on Microfile film and were developed in Microdol developer.

Experimental data. A. *Growth Rate. Rana pipiens*: Frog larvae placed in the experimental medium at stage 11 and left for 7 days showed no appreciable change in growth rate. By the 9th day of exposure, however, the experimentals showed an average length 2 mm less than that of the controls and on the 11th day, the experimentals had shown no further growth while the controls had continued to grow to an average of about 15 mm. Growth, therefore, was completely inhibited by 9 days after the initial exposure of the larvae to DDT.

Fundulus heteroclitus: Fish larvae were subjected to the experimental medium at stage 8 and at stage 29, were allowed to hatch and survive for 6 days beyond hatching and showed no evidence of any effect on their growth.

B. *Deformities. Rana pipiens*: Beginning the 9th day after subjection to the experimental medium of DDT, the larvae showed a narrowing of the trunk region and on the 11th day there developed a constriction just posterior to the operculum and a lateral acute bending of the tail at the point of junction with the body (Plate I, Fig. 1 to 3). These deformities were observed in the experimental larvae almost without exception. There developed a degree of respiratory paralysis in

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[*] Now at Radiological Research Laboratory, Columbia University.

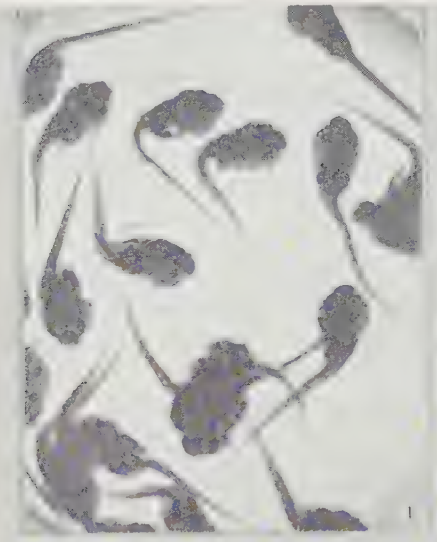
[†] Submitted in partial fulfillment of the requirements for the M.A. Degree at Washington Square College of New York University.

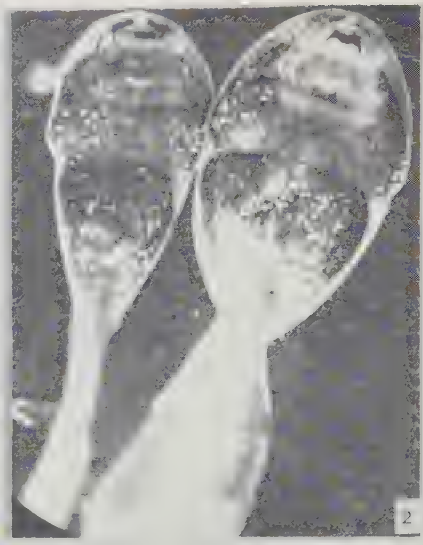
[1] Ellis, M. M. *et al., Science* 1944, **100**, 477.

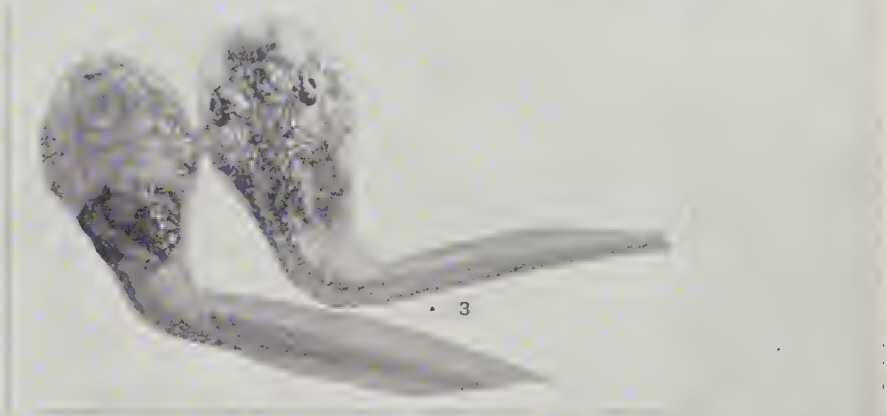
[2] Nelson, A., *et al., Public Health Rep.*, 1944, **59**, 1009.

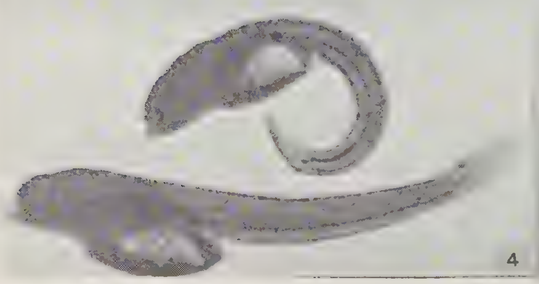
[3] Shumway, W., *Anat. Rec.*, 1940, **78**, 139.

[4] Oppenheimer, J., *Anat. Rec.*, 1937, **68**, 1.









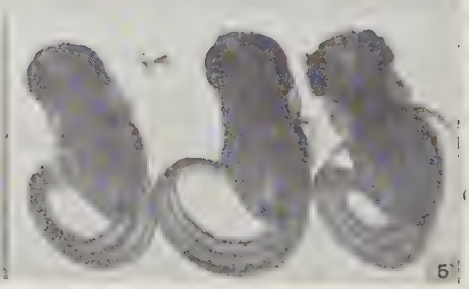


PLATE 1.

Tail curvature of *Rana pipiens* and *Fundulus heteroclitus* larvae following exposure to saturated solutions of DDT.

FIG. 1. Larvae of *Rana pipiens*, placed in a saturated solution of DDT at stages 11 and 12, after 11 days of treatment.

FIG. 2. Larvae of *Rana pipiens*, placed in a saturated DDT solution at stages 11 and 12, after 9 days of treatment. The experimental animal on the left shows an impairment of growth as compared with the control animal on the right.

FIG. 3. Higher magnification of larvae from some experimental group as larvae in Fig. 1.

FIG. 4. Larva of *Fundulus heteroclitus* (above) showing a ventral curvature of the tail upon hatching from a saturated DDT solution, in which it was immersed for 19 days, treatment beginning at stage 8; control (below) untreated.

FIG. 5. Larvae of *Fundulus heteroclitus* showing lateral curvature of tail upon hatching from a saturated DDT solution in which they were immersed for 19 days, treatment beginning at stage 8.

which the mouth was held open but the larvae showed none of the usual respiratory movements.

Larvae introduced into the experimental medium at stages 16, 18, 19 and 25 also showed these deformities but their onset was slower, the older the larvae.

Fundulus heteroclitus: Larvae introduced into the experimental medium at stage 8 hatched at the same time as the controls but upon hatching the experimentals exhibited an acute ventral bending of the tail in more than 80% of the larvae, (Plate I Fig. 4 and 5). There was also a slight constriction just below the operculum, and the mouth was held open (as with *Rana pipiens larvae*) without related respiratory movements.

When Fundulus larvae were introduced into the experimental medium at stage 29, they exhibited mild cases of lordosis and kyphosis by the 6th day post hatchings but there was no bending of the tail shown by larvae exposed earlier and for longer periods.

C. *Mortality*. The mortality rate of the experimentals was more than twice that of the controls for both *Rana pipiens* and for *Fundulus heteroclitus* larvae. Only 75 out of 400 *Rana pipiens* larvae and 35 out of 150 *Fundulus* larvae survived the treatment.

D. *Neuro-Muscular responses. Rana pipiens*: Using the race-track method of Detwiler[5] the degree of response to tactile stimulation at different stages in DDT treatment was determined. A decrease in motor activity was first noted on the 7th day of exposure, was considerably more pronounced on the 9th day and almost extinct by the 11th day (see Fig. 1). On the 11th day the responses to tactile stimulation consisted of generalized fine tremors and an occasional initial sharp single contraction of the tail musculature. There was a definite decrease in muscle tonus and in the ability to assume a normal position.

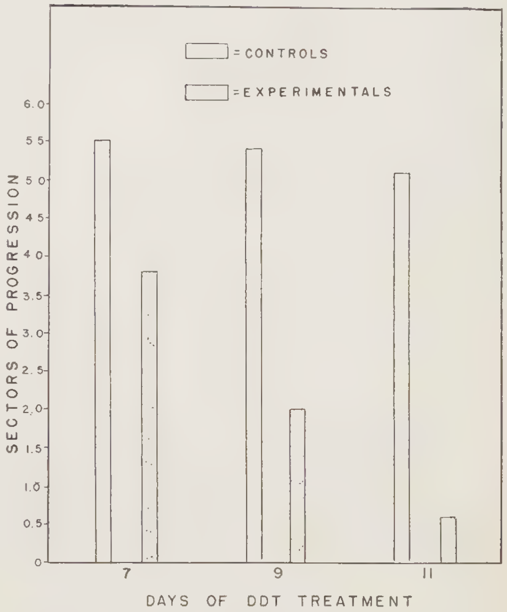


FIG. 1.

Neuro-muscular responses of *Rana pipiens* larvae to tactile stimulation following exposure to saturated solutions of DDT.

Fundulus heteroclitus: Larvae exposed to the experimental medium showed a slight decrease in motor responses to tactile stimuli as early as the fourth day after hatching, even though there was no morphological evidence of damage. There was marked decrease in the response by the 6th day, the larvae showing very much reduced activity and occasionally developing spasmodic contradictions of the trunk and tail musculature, and general fine tremors throughout the body without appreciable forward progression. There was no swimming activity.

E. *Recovery. Rana pipiens*: Larvae subjected to the DDT medium at stage 11 were removed at daily intervals from the 2nd to the 11th day after initiation of the treatment, and placed in the normal control medium. Complete recovery was effected in those larvae which were returned to the normal medium up to and including the 6th day of treatment. Beyond the 6th day of exposure, the larvae retained all of the morphological and physiological symptoms present at the time of removal from the experimental medium, but without further effects.

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[5] Detwiler S. R., *J. Exp. Zool.*, 1946, **102**, 321.

Fundulus heteroclitus: Larvae which were removed from the experimental DDT medium on the 16th day after immersion at stage 8 retained the same deformities and abnormalities in behavior as the experimentals which were allowed to hatch in the DDT medium. Prior to the 16th day there was little or no retention of damage effects.

Discussion. The exact mechanism of DDT action on the living system is still unknown, except that the findings of this investigation corroborate earlier findings on higher forms that the effect is primarily on the neuro-muscular mechanism.

Ellis *et al.*,[1] and Odum and Sumerford,[6] found that cold-blooded animals are more easily affected than are the warm-blooded animals. Ellis *et al.*[1] fed DDT to goldfish in pellet form and the total mortality was approximately 55%. The fish were hyperirritable, and then developed muscular incoordination, muscular spasms, and finally marked prostration during which time the fish lay on its side, breathing irregularly and manifesting convulsive movements. These symptoms were also found in the present investigation in the larvae of *Fundulus heteroclitus*.

The dose required to produce symptoms of toxicity varies with different animals. Odum and Sumerford,[6] found that the median lethal dose for goldfish was 0.1 ppm, for Gambusia was 0.01 ppm and for Culex larvae was 0.0001 ppm. Ginsburg,[7] stated that mosquito larvae fed on DDT were not poisonous to goldfish, but that DDT in solution was definitely toxic.[7] Concentrations of DDT at 1 part in 10,000,000 caused 40% mortality while 1/20,000,000 seemed to be non-toxic.

The concentration of DDT in solution is very difficult to regulate or determine. It was found possible to increase the concentration to 4 ppm by treating the mixture ultrasonically, but the control medium, also treated ultrasonically, was somewhat toxic to the larvae. Based upon rough calculations in relation to the amount introduced and the amount undissolved, and to observations of others, it was estimated that the concentrations used amounted to at least 1 ppm. For purposes of duplication of experimental procedures, however, it can be stated that the concentrations used were saturated at laboratory temperatures of 23° to 25°C, and that this probably represented 1 ppm.

Recently Oppenheimer[8] found similar damaging effects on the larvae of *Fundulus heteroclitus* following subjection to dilute solutions of metrazol. The sharply angled tails appeared as identical morphological effects of DDT and metrazol, and it is entirely possible that the two compounds effect the neuromuscular apparatus of the larvae in much the same manner.

Conclusions. 1. A saturated solution of DDT (estimated at 1 ppm) in spring water for *Rana pipiens* larvae and in distilled water for *Fundulus heteroclitus* larvae, was sufficiently toxic to produce morphological deformities of a similar nature, the most obvious of which was the sharply bent tail and the very much reduced neuro-muscular response to tactile stimulation.

2. The initial evidence of damage by DDT appeared after 7 days of treatment of *Rana pipiens* larvae (beginning at stage 11) and after hatching of *Fundulus heteroclitus* larvae (treatment beginning at stage 8). There was a considerable lag in the effect, due, in all probability, to the fact that DDT is relatively insoluble and therefore difficult to assimilate by the larvae.

3. In addition to the bent tail, there was a gradual decrease in neuro-muscular responses as determined by the Detwiler racetrack method following tactile stimulation, in both Rana and Fundulus larvae. Ultimately the larvae responded with only muscular tremors.

4. The toxic symptoms were not manifest in Rana larvae if they were returned to the control medium after 6 days of subjection to

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[6] Odum, E. P., and Sumerford, W. T., *Science*, 1946, **104**, 480.

[7] Ginsburg, J. M., *J. Econ. Ento.*, 1945, **40**, 475.

[8] Oppenheimer, J. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **66**, 49.

the DDT or in Fundulus as late as 16 days in the medium if subjected at stage 8.

5. There was no effect of DDT on the growth rate of Fundulus larvae, but after 9 days of exposure of Rana larvae they show a definite inhibition of growth.

6. The older the larvae at the time of DDT exposure, the longer the period before the onset of deleterious symptoms.

16952

Biochemical Studies on Livers of Chicks Receiving Graded Levels of Pteroylglutamic Acid.*

JOHN R. TOTTER, WILLIAM E. MARTINDALE, MARION MCKEE, CECELIA K. KEITH, AND PAUL L. DAY.

From the Department of Biochemistry, School of Medicine, University of Arkansas, Little Rock, Ark.

In a recent communication[1] it was shown that the apparent xanthine oxidase activities of livers from chicks fed a purified diet were inversely related to the pteroylglutamic acid (PGA) content of the diet. The livers from the same experimental chicks were also studied for their nucleic acid and PGA content and for their apparent conjugase activity; the values so obtained are reported here.

Experimental. The diets and the methods of handling and care of the chicks are given fully in the previous report.[1] PGA was determined microbiologically by use of *Streptococcus faecalis*.[2] Conjugase was determined by the method of Laskowski, Mims, and Day[3] using Difco yeast extract as the substrate. Since there may be several conjugase inhibitors[4-7] in a mixture such as that used in these tests the absolute values may not be valid, but there seems to be no reason why such a method is not satisfactory for comparative purposes when using a single batch of substrate and the same organ from each bird.

Pentose nucleic acid and desoxypentose nucleic acid were determined on aliquots of 1:5 liver brei employing the methods outlined by Schneider.[8,9]

Results and discussion. The results of the nucleic acid determinations are given in Table I. As indicated earlier,[1] the livers of chicks were relatively larger when the diet was PGA-deficient. The data are arranged to show the nucleic acid content per gram of liver, the total nucleic acid per chick, and the amount per 100 g of chick. The values ob-

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[1] Keith, C. K., Broach, W. J., Warren, D., Day, P. L., and Totter, J. R., *J. Biol. Chem.*, 1948, **176**, 1095.

[2] Mitchell, H. K., and Snell, E. E., Univ. of Texas Pub., 1941, No. 4137, 36.

[3] Laskowski, M., Mims, V., and Day, P. L., *J. Biol. Chem.*, 1945, **157**, 731.

[4] Bird, O. D., Robbins, M., Vandenbelt, J. M., and Pfiffner, J. J., *J. Biol. Chem.*, 1946, **163**, 649.

[5] Sims, E. S., and Totter, J. R., *Fed. Proc.*, 1947, **6**, 291.

[6] Mims, V., Swenseid, M. E., and Bird, O. D., *J. Biol. Chem.*, 1947, **170**, 367.

[7] Hodson, A. Z., *Arch. Biochem.*, 1948, **16**, 309.

[8] Schneider, W. C., *J. Biol. Chem.*, 1945, **161**, 293.

[9] Schneider, W. C., *Cold Spring Harbor Symposia on Quantitative Biology*, 1947, **12**, 169.

TABLE I.

Desoxypentose Nucleic Acid and Pentose Nucleic Acid in Livers of Chicks Receiving Graded Levels of Pteroylglutamic Acid.

Diet	No. of chicks	Liver desoxypentose nucleic acid: Avg, mg/g	Range, mg/g	Per chick, mg	Per 100 g chick, mg	Liver pentose nucleic acid: Avg, mg/g	Range, mg/g	Per chick, mg	Per 100 g chick, mg
Basal	14	2.5	2.1-3.5	14.6	9.6	6.0	3.2-7.8	38.6	27.3
” + 5 μg PGA/100 g	4	3.2	2.9-3.9	20.0	11.3	6.8	5.4-8.4	41.6	34.3
” + 10 ” ” ”	8	3.0	2.3-3.7	22.1	8.1	7.6	4.9-9.9	56.8	35.8
” + 20 ” ” ”	7	2.9	2.2-3.8	23.1	5.5	6.9	5.0-8.2	55.4	26.6
” + 40 ” ” ”	9	3.1	2.0-4.9	26.7	4.3	6.5	4.3-7.9	58.8	27.4
” + 80 ” ” ”	9	3.3	2.4-4.2	30.2	4.8	7.0	4.8-8.0	65.3	23.4
” + 200 ” ” ”	8	3.1	2.1-4.6	34.3	3.8	6.5	4.6-7.9	70.5	23.1
” + 1000 ” ” ”	8	3.3	2.9-3.7	27.5	4.6	6.9	3.9-8.5	58.6	17.8
Commercial diet*	8	3.5	2.6-3.8	28.0	4.4	6.8	4.9-8.4	56.0	17.3

* Purina “Startena,” containing 175 μg of total PGA per 100 g, as shown by assay with *S. faecalis* and suitable conjugases.

tained agree satisfactorily with those obtained by Schneider on rat liver.[6]

The possibility that pteroylglutamic acid may be necessary for thymine synthesis and presumably, therefore, for synthesis of thymine-containing nucleic acids is emphasized in current theories concerning mechanism of action of this vitamin.[10-12] It is of special interest therefore to compare the thymonucleic (desoxypentose nucleic) acid contents of the livers of chicks on the basal diet with those receiving adequate amounts of PGA. As seen from Table I, there is a somewhat lowered desoxypentose nucleic acid content per gram of deficient chick liver as compared with livers from the other groups. However, groups receiving amounts of PGA entirely inadequate (5 μg, 10 μg) for prevention of deficiency symptoms nevertheless have values of desoxypentose acid per gram of liver equal to or greater than those receiving more than enough of the vitamin. When calculated on the basis of total liver nucleic acid per 100 g of chick, those on the basal diet have greater amounts than the ones receiving excess of PGA (200 μg, 1000 μg). If it may be assumed that the method used is specific for thymine-containing nucleic acids, the data do not offer strong support to the view that PGA deficiency blocks the synthesis of thymine. In this connection it may be recalled that Davidson[13] has found that both types of nucleic acid are elevated in the bone marrow of untreated pernicious anemia patients. Successful treatment of his patients was followed by a reduction to normal of their marrow nucleic acids.[13] It is difficult to reconcile these results with the hypothesis that the sole action of PGA is to promote thymine synthesis.

The pentose nucleic acid content was more uniform than the desoxypentose nucleic acid content. The level of pentose nucleic acid in the livers of the totally deficient groups was

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[10] Stokes, J. L., *J. Bact.*, 1944, **47**, 433.

[11] Spies, T. D., Vilter, C. F., Cline, J. K., and Frommeyer, W. B., *Southern Med. J.*, 1946, **39**, 269.

[12] Wright, L. D., Skeggs, H. R., and Huff, J. W., *J. Biol. Chem.*, 1948, **175**, 475.

[13] Davidson, J. N., *Cold Spring Harbor Symposia on Quantitative Biology*, 1947, **12**, 50.

only slightly lowered when compared with the other groups; the difference is probably not statistically significant.

According to Mims *et al.*,[6] desoxypentose nucleic acid is a potent inhibitor of conjugase activity. It might therefore be supposed that any alterations in the level of this nucleic acid would be reflected inversely by changes in the conjugase activity of the liver samples. In Table II are shown the apparent conjugase values at pH 7 and pH 4.5 as well as the free and total PGA content of the livers; the total PGA was determined with chicken pancreas conjugase at pH 7 and hog kidney conjugase at pH 4.5. There seems to be no particular relation between the conjugase activity and either the PGA intake or the desoxypentose nucleic acid content of the livers. It is interesting that the only group of chicks receiving conjugated PGA, those on the commercial diet, exhibited the lowest liver conjugase activity. The degree of reduction, however, is of doubtful significance.

The free PGA content of the livers appears to be about equal to the total PGA, *i.e.* little additional vitamin was liberated by treatment with added conjugase from either chicken pancreas or hog kidney. In a few cases the free is shown as greater than total PGA; these occurrences are undoubtedly due to cumulative errors in the assays.

The average contents of PGA in the livers of the various groups provide evidence from which the minimum requirement for the vitamin may be estimated. It is interesting that the liver contents of PGA in the chicks up to the group receiving 40 μg per 100 g of diet show little change, while the 80 μg group and those receiving more than 80 μg are 2 to 3 times higher than the deficient groups in this respect. From this it appears that the minimum requirement for protection from deficiency should be set at a value not lower than 40 μg per 100 g of diet. This is in agreement with the estimate from the growth curve of these chicks published earlier.[1] Estimation from blood cell numbers, however, indicates the minimum value to be somewhat lower when calculated for red cell values. On the contrary, white cell (chiefly lymphocytes)[14,15] numbers and thymus weights[15]

TABLE II.
Average Contents of Pteroylglutamic Acid and Conjugase in the Livers of Chicks Receiving Graded Levels of Pteroylglutamic Acid.

		Free PGA autolyzed at		Total PGA		Apparent liver conjugase activity*	
				Hog kidney conjugase	Chicken pancreas conjugase		
	No. of chicks	pH 4.5 μg/g	pH 7.0 μg/g	pH 4.5 μg/g	pH 7.0 μg/g	pH 4.5 units/g	pH 7.0 units/g
Basal	12	2.0	1.5	2.1	1.9	3.1	6.4
,, + 5 μg PGA/100 g	4	1.3	1.5	1.1	1.5	2.9	7.4
,, + 10 ,, ,, ,,	8	1.3	2.0	2.5	3.1	4.2	12.3
,, + 20 ,, ,, ,,	7	2.2	2.2	3.2	3.0	3.7	10.6
,, + 40 ,, ,, ,,	9	2.9	2.3	2.9	3.2	2.7	6.3
,, + 80 ,, ,, ,,	9	8.5	8.7	9.9	8.6	4.1	8.9
,, + 200 ,, ,, ,,	9	5.0	5.1	6.0	6.3	5.1	12.5
,, + 1000 ,, ,, ,,	8	7.7	7.3	7.8	8.9	3.3	8.2
Commercial diet†	8	6.7	5.3	7.3	7.1	2.1	6.2

* A unit of conjugase activity is expressed here as the μg of PGA liberated in 4 hours by 0.2 ml of 1:20 liver brei acting on 80 mg of Difco yeast extract in a total volume of 2 ml.

† Purina "Startena," containing 175 μg of total PGA per 100 g, as shown by assay with *S. faecalis* and suitable conjugases.

increased logarithmically with increasing levels of PGA to as high as 200 μg per 100 g of diet.

The concentrations of PGA in the livers of the chicks also provide some evidence concerning the mode of action of the vitamin in reducing the apparent liver xanthine oxidase activity. *In vitro* tests of the sample of PGA used in these experiments indicated that a concentration of approximately 40 μg per ml would be needed to reduce the xanthine oxidase activity to one-half its initial value.[†] The relatively low levels of PGA encountered in all of the liver samples indicate that either the impurity probably responsible for the *in vitro* anti-xanthine oxidase activity[16] is better retained than is PGA, or that some other mechanism than the accumulation of this substance is responsible for the reduced activity found in our previously published experiments.[1] It is conceivable that the xanthine oxidase is inhibited or reduced by a metabolic product of PGA normally produced in relatively large quantity.

Summary. Determinations of desoxypentose nucleic acid and pentose nucleic acid, pteroylglutamic acid (PGA), and conjugase have been made on the livers of chicks receiving 0, 5, 10, 20, 40, 80, 200, and 1000 μg of PGA per 100 g of diet and on controls receiving a commercial diet. Desoxypentose nucleic acid was found to be somewhat low in the livers of the negative control group of chicks but differences between all other groups were of doubtful significance.

The liver conjugase levels of the various groups, whether determined at pH 4.5 or at pH 7.0, were found to be unrelated to the dietary intake of PGA and to the nucleic acid content of the livers.

The liver PGA freed by autolysis was approximately the same as that determinable after treatment with either chick pancreas conjugase or hog kidney conjugase. The levels of PGA found in the chick livers indicate that storage of excess vitamin did not take place until the dietary level exceeded 40 μg per 100 g.

The bearing that the findings have on the mode of action of dietary PGA in reducing liver xanthine oxidase is briefly discussed.

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[14] Campbell, C. J., McCabe, M. M., Brown, R. A., and Emmett, A. D., *Am. J. Physiol.*, 1945, **144**, 348.

[15] Unpublished data from this laboratory.

[†] Experiments conducted *in vitro* indicate that 2 mols of the xanthine oxidase inhibitor react with one mol of chick liver xanthine oxidase while only one mol reacts per mol of rat liver xanthine oxidase. That is, the degree of inhibition using chick liver is proportional to the square of the concentration of added PGA while it is proportional to the logarithm of the PGA concentration when using rat liver as the source of xanthine oxidase.

[16] Kalckar, H. M., Kjelgaard, N. O., Klenow, H., *J. Biol. Chem.*, 1948, **174**, 771.

16953 P

Influence of Pteroylglutamic Acid Administration Upon Fecal Riboflavin Values in Human Subjects.[*]

LURA MAE ODLAND, DOLORES M. OTTO,[†] AND HELEN T. PARSONS.

From the Department of Home Economics, College of Agriculture, University of Wisconsin, Madison.

In the course of investigations in this laboratory on the metabolism of riboflavin in human subjects on weighed, uniform diets of

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[*] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation; and by a commercial grant from the Jos. Schlitz Brewing Company of Milwaukee.

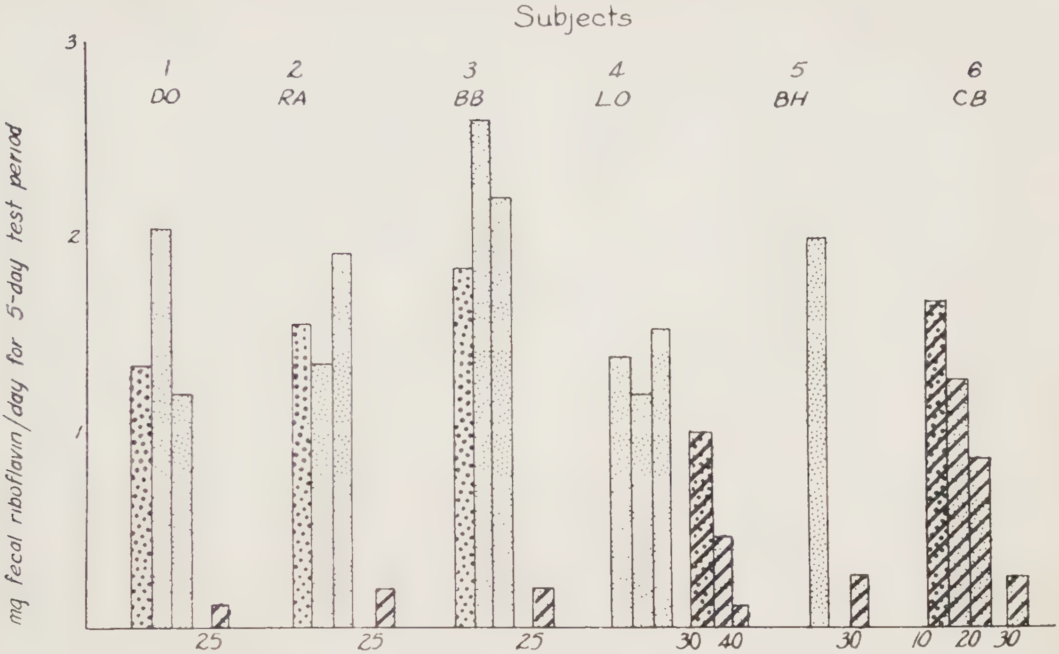

Fig. 1.

The progressively depressing effect of the daily administration to human subjects of 10 mg doses of pteroylglutamic acid on fecal riboflavin values.

Small dots: basal diet; larger dots: self-chosen diet; ////: 10 mg dose pteroylglutamic acid; numerals on the abscissa indicate number of days dose prior to fecal collection.

known riboflavin content[1-3] doses of pteroylglutamic acid were incorporated in certain of the experimental diets.

Experimental. Six university women in a satisfactory nutritional state were maintained on an adequate uniform weighed basal diet providing a daily intake of 3.8 mg of riboflavin with and without the addition of 10 mg of synthetic pteroylglutamic acid. This dose is considered to be within the lower part of the therapeutic range.[4] For the first two basal periods of Subject 4, the diet was identical with one reported earlier[1] and for all other subject-periods this diet was modified only by the addition of 50 g of meat loaf, thus increasing the protein content of the diet to 84 g. For securing additional data, fecal riboflavin values for 5-day periods were also determined on self-chosen diets.

For conformity with other studies, certain supplements were added to the basal diet[‡] as follows: Subjects 1, 2 and 4 received 14 g dried brewers' yeast daily, and Subjects 3, 4 and 6 received supplements[‡] of 2.0 mg thiamine, 0.45 mg riboflavin, 5.24 mg niacin, 50.4 mg choline, 0.14 mg pyridoxine, 1.0 mg calcium pantothenate, and 10 μg pteroylglutamic acid with the exception of the first 2 periods of Subject 4.

Five-day fecal collections were homogenized in a Waring Blendor and preserved in acid-alcohol. Riboflavin was determined by the fluorometric method of Conner and Straub.[5] Duplicate determinations were made

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[†] Present address: Department of Home Economics, University of California, Berkeley, Calif.

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[‡] Thanks are due to the Lederle Laboratories, Inc., Pearl River, N. Y., for the synthetic pteroylglutamic acid (Folvite); to Hoffman La Roche, Nutley, N. J., for other vitamin supplies; and to the Pineapple Research Institute of Hawaii for the gift of specially packed crushed pineapple.

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on separate days for all samples. Food aliquots representing 25% or 50% of the weighed daily food intake, except the self-selected diets, were homogenized and assayed for riboflavin.

Results and discussion. The fecal riboflavin values observed during the control periods are consistent with other data from this laboratory for subjects ingesting essentially the same basal diet. Similar fecal riboflavin concentrations are also reported by Hathaway and Lobb[6] for subjects ingesting a natural diet containing 1.33 mg of riboflavin.

Upon supplementation of the diets of the subjects with 10 mg of pteroylglutamic acid daily for periods of 25 to 40 days, regular and consistent decreases were observed in fecal riboflavin. Values comparable to the lower range in this series have been noted in this laboratory only in one other instance, *i.e.*, when subjects were consuming a diet composed of milk with ascorbic acid and mineral supplements.[7]

Darby *et al.*[8] have noted that certain deficiency states due to decreases in gastrointestinal absorption may be relieved by the administration of pteroylglutamic acid. These observations suggest that in the present experiment, certain vitamin interrelationships involving the absorption of riboflavin might be concerned in the decrease in fecal riboflavin in the presence of long-continued daily dosage of 10 mg of pteroylglutamic acid. Another obvious possibility is that administration of pteroylglutamic acid measurably influences the character of the intestinal flora, resulting in a decrease in riboflavin synthesis, an increase in riboflavin destruction or both. The nature of this pronounced change in fecal riboflavin is being investigated; these results are being reported at this time because of their possible clinical implications.

Summary. As a part of long-time experiments in this laboratory on riboflavin metabolism in human subjects on weighed diets of known riboflavin content, it was noted that with the daily administration of 10 mg of synthetic pteroylglutamic acid for 25 to 40 days there was a striking decrease in fecal riboflavin values as compared to basal periods.

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16954

Effect of Adrenal Cortical Extract on the Blood Picture and Serum Proteins of Fowl.

ANSELL B. SHAPIRO AND A. M. SCHECHTMAN.
From the Department of Zoology, University of California, Los Angeles, Calif.

Preliminary to studies on the effects of adrenal cortical extract (ACE) on embryonic development of the chick's blood, it became desirable to know how the blood elements of the adult fowl respond to cortical substances. We have been unable to find pertinent data for the fowl; furthermore the results obtained with various mammals are not consistent. Absolute peripheral lymphopenia has been observed in the normal mouse,[1] rat,[1,2,3] rabbit,[1] dog,[2] and in man[4,5] after injections of pituitary adrenotrophic hormone, and in the mouse,[1] rat,[1,6] rabbit,[1] and man[1,4,5] after adrenal cortical substances. However, others[3,7] have reported little or no change in the blood cell picture of the rabbit after therapy with ACE. The normal rat[8] and cat[9] have been shown to respond to pituitary adrenotrophic hormone therapy with a marked decline in number of thoracic duct lymphocytes. On the other hand Valentine *et al.*[10] observed no change in number of thoracic duct lymphocytes in cats injected with ACE.

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[1] Dougherty, T. F., and White, A., *Endocrinology*, 1944, **35**, 1.

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[3] Yoffey, J. M., and Baxter, J. S., *J. Anat.*, 1946, **80**, 132.

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The relationships of hypercortico-adrenal activity to serum protein levels in mammals is likewise controversial. Dougherty and White[11] report such activity to be associated with elevation in total serum protein in mice and rats. In the rabbit there is an increase in serum globulin due to rise in beta and gamma globulin.[12] In rats a decrease in gamma, but an increase in alpha and beta globulin, after ACE therapy has been reported.[13] Li and Reinhardt[14] found no effect of pituitary adrenotrophic hormone on plasma globulins in the normal rat, and Eisen *et al.*[15] detected no change in gamma globulin concentration after repeated injections of ACE, although a single injection did raise the serum antibody nitrogen in immunized rabbits.

It is our purpose in the present paper to report changes in the blood picture of 2 breeds of fowl at various periods after a single injection of ACE. Some observations on serum protein levels are included.

Method. Adult Single Comb White Leghorn laying hens and New Hampshire Red non-laying pullets were used. The Leghorns were 7 months of age at the outset of the experiment and weighed 1800 to 2200 g. The pullets were 3½ months old and weighed 1800 to 2200 g. The animals were housed outdoors in wire bottom cages protected from wind, dampness, and rain, and were maintained on commercial diets.

Blood was obtained from the ulnar vein and the smears were usually stained within 24 hours using Wright's and Giemsa, the latter diluted tenfold. The differential white blood cell determinations were made by counting 300 leucocytes, and occasionally 400, in representative regions of each slide.

Blood for both total leucocyte and erythrocyte counts was diluted 100 times[16] in a certified red blood cell diluting pipette with a solution[17] composed of 25 mg of neutral red added to 100 ml of 0.9% saline, and the cells were identified in a Neubauer Brightline Counting Chamber.

Total protein of the sera was estimated with the biuret reagent of Weichselbaum.[18] Optical densities of the sera were determined at 555 mu on a model 11A Coleman spectrophotometer by subtracting the optical density readings of serum-containing cuvettes from those with saline prepared at the same time. The density readings are thus simply measures of the relative total protein content rather than of absolute amounts.

Precipitation of total serum globulin was carried out with the ammonium sulfate method of Fine.[19] Nine and a half ml of 27.79% ammonium sulfate was added to 0.5 ml of serum placed in a 15 ml conical centrifuge tube, giving a final concentration of 2M ammonium sulfate. The tubes were refrigerated overnight and centrifuged the next

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morning at 2500 r.p.m. for ½ hour. The supernatant, containing mainly albumin and ammonium sulfate, was separated from the packed precipitate by decantation, and the tubes were inverted for 1½ hours on filter paper to remove excess liquid. The globulin was dissolved in 0.3 ml of 0.85% saline. To 1/10 ml of this protein solution in a cuvette were added 4.9 ml of 0.85% saline and 5.0 ml of biuret reagent. Optical density readings were made at 555 mu on the spectrophotometer. Each serum sample was precipitated and tested in duplicate.

The adrenal cortical extracts (aqueous)* and sterile 0.85% saline were injected subcutaneously in the pectoral region. Immediately prior to the injections blood counts were taken from each animal and are designated in the tables as "0 hour" counts.

Experiments and Results. (1) *White Leghorn laying hens.* A single injection of 2 ml of ACE (Table I) was followed by a marked leucopenia, pronounced absolute lymphopenia, decrease in number of erythrocytes and an absolute polymorphonuclear leucocytosis. The severe lymphopenia, indicated by a post-injection mean of ½ the pre-injection mean at 0 hour accounts for the leucopenia. Differential counts revealed a 11.7% decrease in lymphocytes and a concomitant increase of 11% in polymorphonuclear cells 3 hours following injection. The latter change was apparently due mainly to heterophils. All changes were maximal at 3 hours after injection except for the absolute granulocyte alteration, which was maximal at 6 hours. The blood picture returned to normal by the end of 24 hours. The absolute and differential alterations are statistically significant; furthermore all animals showed the absolute changes, and only one hen failed to reveal the percentage deviations.

The effects of saline injections, of a 24-hour fast (with water *ad libitum*), and of fasting plus saline are shown in Table II. These procedures caused no appreciable alteration in the blood picture. The effect of fasting

TABLE I. Effect of a Single Injection of 2 ml of ACE* on Blood Cell Levels in 11 White Leghorn Laying Hens.

		Cells per cu. mm				%				
	Hr	RBC × 1000	WBC	Lymphocyte	Polymorph	Lymphocyte	Monocyte	Eosinophil	Basophil	Heterophil
Mean	Pre-0†	2,714	27,118	20,908	4,068	77.1	7.9	1.5	1.6	11.9
S.D.‡		243	4,288	3,109	1,792	4.8	2.1	0.6	0.8	5.0
Mean	0	2,738	30,727	23,352	5,715	76.0	5.4	0.8	1.1	16.7
S.D.		325	5,656	5,098	2,794	8.4	2.0	0.7	0.9	7.0
Mean	1	2,448	23,383	17,257	4,887	73.8	5.3	0.3	1.4	19.2
S.D.		274	6,862	5,664	3,132	7.9	2.4	0.3	0.9	7.6
Mean	3	2,367	18,378	11,817	5,440	64.3	6.1	0.4	1.5	27.7
S.D.		256	7,061	5,131	3,173	11.4	2.4	0.5	1.1	11.3
Mean	6	2,492	26,879	17,794	7,768	66.2	4.9	0.4	1.2	27.3
S.D.		238	6,967	5,843	2,858	10.0	2.5	0.4	0.9	9.1
Mean	9	2,588	30,107	21,737	7,165	72.2	4.0	0.5	0.8	22.5
S.D.		300	6,163	5,471	3,235	11.3	1.5	0.5	0.6	9.4
Mean	24	2,624	29,573	23,274	4,968	78.7	4.5	0.7	1.6	14.5
S.D.		447	7,875	4,562	3,247	10.4	2.2	0.6	1.2	8.4

* Wilson's product injected into 9 hens and Upjohn's into 2 hens.

† Derived from 40 counts made on the same 11 hens during the 4 weeks preceding injection.

‡ Standard deviation of the mean; S.D. = $\frac{\sqrt{\Sigma d^2}}{\sqrt{n-1}}$

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* We are indebted to Dr. David Klein of the Wilson Laboratories and to Dr. W. F. Wenner of the Upjohn Company for supplying us with their adrenal cortical products.

TABLE II.

Effects of Saline, of Fasting, of Saline After Fasting, and of ACE* After Fasting on the Blood Picture of White Leghorn Laying Hens.

	No. of hens	Hr.	Cells per mm³				%				
			RBC × 1000	WBC	Lymphocyte	Polymorph	Lymphocyte	Monocyte	Eosinophil	Basophil	Heterophil
Inj. of 2 ml of saline											
Mean	3	0	2,507	26,917	20,457	5,087	76.0	5.1	2.1	1.3	15.5
S.D.			249	4,653	3,253	1,274	3.0	1.0	0.9	0.8	3.9
Mean		3	2,703	24,917	19,012	4,460	76.3	5.8	1.7	1.9	14.3
S.D.			255	4,404	2,244	1,630	4.2	1.1	1.2	0.9	2.3
Mean		6	2,497	26,667	20,240	4,987	75.9	5.4	1.3	1.1	16.3
S.D.			255	5,125	3,778	1,708	4.7	0.8	0.6	0.6	4.4
Mean		9	2,635	25,167	18,322	5,863	72.8	3.9	1.7	1.2	20.4
S.D.			320	1,258	702	1,784	6.3	0.8	2.5	0.6	3.3
After 24-hr fast											
Mean	10	0	2,651	26,000	18,850	5,330	72.5	7.0	1.1	1.6	17.8
S.D.			320	5,678	4,286	2,833	10.8	2.9	0.5	0.8	9.0
Mean		24	2,730	25,500	17,570	5,916	68.9	7.9	1.2	2.0	20.0
S.D.			369	5,793	4,521	3,246	12.7	4.6	0.6	1.6	9.6
Inj. of 3 ml of saline after fasting											
Mean	3	0	2,670	23,583	16,720	5,613	70.9	5.3	1.6	2.0	20.2
S.D.			255	1,808	2,234	573	4.3	1.9	1.4	1.2	4.4
Mean		3	2,730	22,750	15,971	5,642	70.2	5.0	1.1	2.1	21.6
S.D.			152	2,750	3,783	1,785	9.9	1.8	1.1	0.2	6.3
Inj. of 3 ml of ACE after fasting											
Mean	10	0	2,334	23,000	17,204	4,623	74.8	5.1	0.9	1.7	17.5
S.D.			326	4,452	4,393	1,396	7.4	1.8	0.7	1.3	6.0
Mean		3	2,270	10,450	5,706	4,096	54.6	6.2	0.2	1.1	37.9
S.D.			263	2,020	1,704	1,075	11.3	2.7	0.3	0.8	10.3

* Product of Wilson Laboratories.

was studied since it was considered desirable to reduce the high lipid content of the sera[20] for electrophoretic analyses.

Two months after the above injection of 2 ml of ACE, the same group of birds was fasted for 24 hours and then given 3 ml of ACE (Wilson). Cell count alterations were similar to those obtained 3 hours after the first injection of 2 ml of ACE except that there was no significant decrease in number of erythrocytes (Table II). The lymphocyte mean was ⅓ the pre-injection mean. In one hen showing an absolute lymphopenia involving a drop from 22,400 to 5,490, an electrophoretic analysis (using veronal buffer)† of the serum showed no significant change in any protein fraction from levels found in the same animal's serum after saline injection 3 days earlier. However, the post-extract serum showed a 15% total serum protein increase.†

(2) *New Hampshire non-laying pullets.* A single injection of 3 ml of saline into 12 New Hampshire pullets did not alter the peripheral blood cell picture significantly (Table III). Ten days after the saline injection, each animal received a single injection of 3 ml of ACE. Three hours after injection there was an absolute lymphopenia (Table III) with a mean of 12,833 compared to the pre-injection mean of 20,466 and a slight drop in the number of erythrocytes. The total leucocyte level remained normal as a result of increase in the number of granulocytes. Differential leucocyte determinations indicate a mean decline of 27.9% in lymphocytes, an increase of 26.3% in polymorphonuclear cells, mainly due to heterophils, and a slight drop in eosinophils.

Although the aforementioned changes in blood cell values are statistically significant, data for total protein variation between post-saline and post-extract levels (Table III) yields a t value of 2.0 which is slightly below the criterion of significance (2.2). If the protein values for individual birds are considered

TABLE III. Effect of 3 ml of ACE (Wilson) on Blood Counts, Total Serum Protein, and Total Globulin Levels in 12 New Hampshire Red Non-laying Pullets (treated 10 days earlier with 3 ml of saline).

			Cells per mm³				%					Serum optical density reading	
		Hr.	RBC × 1000	WBC	Lymphocyte	Polymorph	Lymphocyte	Monocyte	Eosinophil	Basophil	Heterophil	Total protein	Total glob.
Pre-sal.	Mean	0	2,432	30,208	20,511	7,884	67.9	6.0	1.8	3.0	21.3		
	S.D		344	8,818	6,188	4,005	9.8	1.7	1.4	1.1	9.5		
Post-sal.	Mean	3	2,398	28,854	18,669	8,397	64.7	6.2	1.4	3.2	24.5	.425	.200
	S.D.		196	5,303	4,917	3,667	10.6	2.2	0.6	1.2	9.3	.091	.064
Pre-ACE	Mean	0	2,679	27,000	20,466	5,184	75.8	5.0	1.2	2.3	15.7		
	S.D.		390	4,979	4,749	1,357	5.7	2.1	1.0	1.2	4.5		
Post-ACE	Mean	3	2,535	25,792	12,833	12,190	47.9	6.6	0.3	1.9	43.3	.456	.187
	S.D.		313	5,405	3,395	3,631	8.9	2.2	0.4	1.4	8.5	.087	.048

Note: t value for protein change is 2.0, and for globulin 1.2; $t = 2.2$ ($P = .05$) is significant; $t = \frac{x}{s}\sqrt{n-1}$, x = difference in means and $s = \sqrt{\frac{\Sigma(x_i - x)^2}{n}}$ where x_i = difference between the control and experimental value in the same animal.

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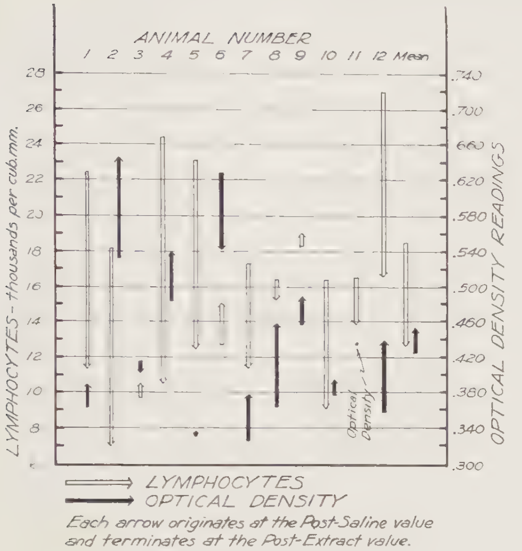
[20] Herrmann, G. R., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 229.

† Performed through the courtesy of Dr. John Mehl, Professor of Biochemistry at the University of Southern California School of Medicine. Total protein determined by a biuret method.



Each arrow originates at the Post-Saline value and terminates at the Post-Extract value.

FIG. 1.

Effect of a single injection of 3 ml of ACE on lymphocyte and total serum protein levels in each of 12 New Hampshire Red non-laying pullets.

(Fig. 1), it is evident that 9 of the 12 pullets showed higher serum protein levels and one showed no change. Despite the borderline t value, this indicates a trend toward elevation in total serum protein.

It is evident from Fig. 1 that increase in serum protein levels is associated with lymphopenia. Of 9 animals revealing a lymphopenia, 8 also showed an elevation in serum protein, and one no change. Two pullets (No. 3 and 6, Fig. 1) deviated from this general picture, showing an apparent decrease in serum protein accompanied by an increase in lymphocytes. In one of these (No. 3) the changes observed are insignificant. While the results suggest a correlation between lymphopenia and serum protein increase in individual pullets, there appears to be no obvious quantitative relationship.

The t value for the difference between globulin readings was 1.2, 6 animals showing a decrease, 5 an increase, and 1 no change after extract injection. We have, therefore, no indication of a consistent globulin change as a result of ACE injection.

Discussion. The different degrees of lymphopenia obtained in the present experiments may be due to an inherent breed difference in reactivity, to differences in age since lymphoid tissue is less active in older birds,[21] or to differences in amounts of injected extract. The overall picture, however, indicates absolute and relative lymphopenia and absolute and relative heterophil leucocytosis occur consistently after a single injection of ACE into both breeds of fowl. Furthermore the maximal lymphopenic effect occurred at 3 hours in the present study on the fowl, which is in good agreement with the work on the rat and rabbit.[1]

The present results suggest an augmentative effect of ACE therapy on total protein levels in fowl. However, we can not be certain that the effect was due solely to the ACE injected inasmuch as 10 days elapsed between bleeding of animals for control and experimental serum protein determinations. The apparent increase in protein level may possibly have been due to 10 days of maturation, although no significant changes were found in the lymphocyte counts. The data of Herrmann[20] suggests that non-laying chickens of 11-22 weeks of age have a serum protein mean of 4.02 ± .5 (S.D.) as compared to 6.1 ± 1.3 for laying hens. Definite conclusions on the effects of ACE on serum protein levels must await more complete data on protein changes in the serum of the normal fowl during the 15th and 16th week of development.

Conclusions. 1. White Leghorn laying hens injected subcutaneously with aqueous adrenal cortical extract, showed leucopenia, absolute and relative lymphopenia and absolute and relative increase in granulocytes attributable mainly to heterophils. The effects were maximal at 3 hours and the blood picture returned to normal by the end of 24 hours.

2. New Hampshire Red non-laying pullets injected with ACE displayed similar changes with the exception of leucopenia which was masked by increase in polymorphonuclear cells.

3. ACE therapy produced no statistically significant increase in total serum protein or serum globulin levels. However, a trend toward increase in total serum protein, accompanied by lymphopenia, is indicated for the majority of the individual birds studied.

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16955

Stabilization of the Thromboplastic Lipid by Hydroquinone.

Joseph Lein and Patricia S. Lein. (Introduced by Robert Gaunt.)

From the Department of Zoology, Syracuse University, Syracuse, N. Y.

The instability of the thromboplastic lipid has been recognized for many years. McLean[1] demonstrated the progressive loss of thromboplastic activity of cephalin and, by concomitantly measuring its degree of unsaturation, came to the conclusion that there was direct relation between activity of cephalin and its degree of unsaturation. Similarly, Hanzlik and Weidenthal[2] showed that cephalin loses activity in 2 months.

In previous studies on the thromboplastic lipid[3] we were faced with the problem of assaying preparations over long periods of time. An effort was made therefore to protect the active agent from autoxidation using various known antioxidants. Studies were carried out with thiourea, ascorbic acid, acetone bisulfite, and hydroquinone. Of these agents, hydroquinone was by far the most effective. Deutsch *et al.*[4] had previously found that hydroquinone inhibited phospholipid oxidation catalyzed by ascorbic acid. Similarly, Rusch and Kline[5] reported the inhibition of phospholipid oxidation by polyphenolic compounds including hydroquinone. Experiments were accordingly undertaken to find the conditions necessary to prevent the autoxidation of the thromboplastic phospholipid and to determine if the thromboplastic activity would be stabilized under these conditions.

Methods. The lipid preparations used were mixtures of lecithin and cephalin isolated from beef brain and found to have considerable thromboplastic activity.[3] The mixture is a crude one and is designated by the term phospholipin for convenience.

The degree of unsaturation was determined by the Hanus iodobromine method (U. S. Pharmacopea, XI), care being taken to insure at least a 60% excess of the reagent. In the case of preparations protected against autoxidation it was found necessary first to remove the hydroquinone by washing with acetone. In all cases iodine numbers are expressed as grams of iodine reacting with 100 g of the phospholipin freed from hydroquinone.

In order to add different quantities of hydroquinone to phospholipin, a known amount of phospholipin was placed in a glass-stoppered cylinder containing 100 ml of acetone saturated with hydroquinone. Additional hydroquinone was then added to give the proportions desired and the mixture shaken well for 15 minutes. It was then filtered through a Büchner funnel and the residue dried *in vacuo*. This procedure was found to be sufficient to yield a homogenous mixture of hydroquinone and phospholipin.

The preparations were assayed for thromboplastic activity by determining the coagulation time of recalcified dog plasma with and without the addition of the thromboplastic agent. From these values the percent decrease in coagulation time produced by the agent was calculated and used as the criterion for thromboplastic activity. The technics used were the same as those described previously.[3] Two determinations were done in each case,

TABLE I.
Stability of Untreated Phospholipin.

Days	pH	Iodine No.	Activity % decrease in coag. time
0	6.49	78.4	64.2
2	6.21	77.7	64.4
5	6.30	70.4	68.4
7	5.83	59.9	61.2
10	5.30	58.3	65.1
12	5.19	55.2	57.2
15	4.93	56.8	32.4

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[3] Hays, H. W., and Lein, J., *Arch. Biochem.*, 1945, **7**, 69.

[4] Deutsch, H. F., Kline, B. E., and Rusch, H. P., *J. Biol. Chem.*, 1941, **141**, 529.

[5] Rusch, H. P., and Kline, B. E., *Can. Res.*, 1941, **1**, 465.

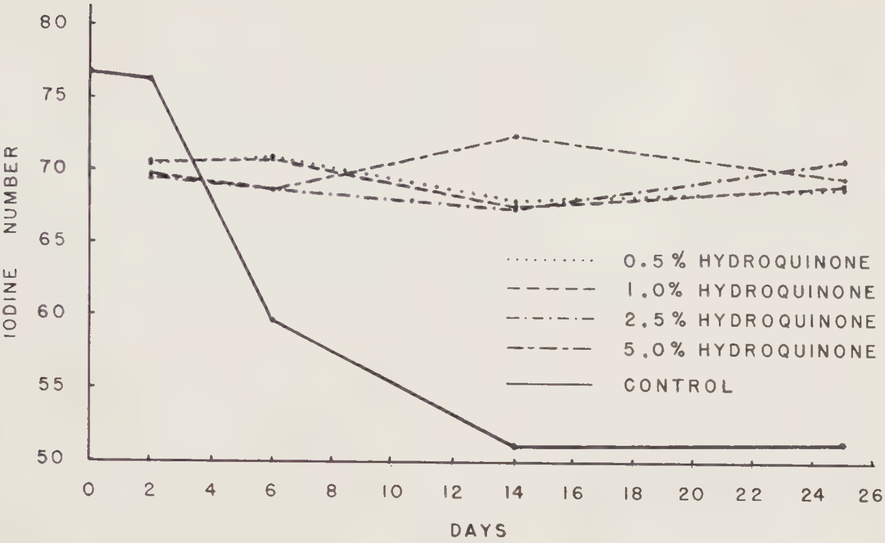

FIG. 1.
The prevention of autoxidation of phospholipin by hydroquinone in concentrations of from 0.5 to 5.0%.

and the average used, providing the deviation did not exceed 10% of the mean. In the few cases where the deviations were greater, additional determinations were carried out.

Autoxidation of Phospholipin. Phospholipin when freshly prepared is granular and has a light cream color. In the course of several days at room temperature and in diffuse light it progressively darkens, becoming orange in color after 7 days, and reddish-brown after 30 days. Concomitant with these color changes, there is a decrease in the pH of saline emulsions of phospholipin, a decrease in its iodine number and a decrease in its thromboplastic activity. Values of these changes in a typical experiment are given in Table I.

The pH values of 0.2% phospholipin emulsions were determined with a glass electrode pH meter. The pH decreased with time, the rate of decrease being greatest at about the tenth day. With a decrease in the pH value, there was a concurrent decrease in iodine number. The iodine number decreased most rapidly between the second and seventh day and tended to reach a stationary level after 12 days. The thromboplastic activity of 0.2% emulsions did not decrease markedly until the twelfth day. This long latent period was in all probability due to the fact that a 0.2% emulsion is in excess of the minimal concentration that would produce maximal activity.[3] Thus, it is evident that phospholipin is very susceptible to autoxidation which markedly alters its physiological as well as chemical characteristics.

Prevention of Autoxidation by Hydroquinone. The amount of hydroquinone added to the phospholipin was expressed as percent of the final hydroquinone-phospholipin mixture. Experiments were carried out using concentrations of hydroquinone varying from 0.5 to 5% and the iodine numbers and thromboplastic activities determined at various time intervals. Parallel determinations were carried out simultaneously on a sample of untreated phospholipin in each series.

Results of the iodine number determinations are presented graphically in Fig. 1. Complete protection against autoxidation by the amounts of hydroquinone used was obtained for 25 days while the untreated control decreased from its original iodine number of 75.7 to a final value of 51.2 in this period of time. It should also be noted that the initial iodine numbers of the hydroquinone-protected phospholipin were considerably lower than that of the control. The reason for this is not apparent but may be due to small amounts of hydroquinone that resist the washings with acetone prior to the iodine number determinations.

With this stabilization of the iodine number there occurred a stabilization of the thromboplastic activity of the preparations. After the 25 day period the percent decrease in coagulation time produced by the control was only 44 while that of the hydroquinone-protected phospholipin was 80, 79, 79, 80 for the respective 0.5, 1, 2.5 and 5% hydroquinone-phospholipin preparations.

Summary. Hydroquinone in concentrations as low as 0.5% protected a thromboplastically active phospholipid preparation from being autoxidized for a period of at least 25 days. With this protection against autoxidation there was a protection against loss of thromboplastic activity.

16956

Metabolism of Isocysteine, Labeled with Radiosulfur (S^{35}), in the Rat.

D. D. DZIEWIATKOWSKI* AND W. J. WINGO.† (Introduced by B. B. Wells.)

From the Departments of Biochemistry, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Md., and University of Texas Medical Branch, Galveston, Texas.

The administration of isocysteine hydrochloride, α-thiol-β-amino-propionic acid, to rabbits results in a large excretion of "extra" sulfur in the urine.[1] Almost all of this extra sulfur is in organic combination; a large fraction of it is in the form of disulfide which is presumably isocystine, since it does not give the Sullivan[2] reaction. When isocysteine is fed to rats maintained on a diet low in proteins (6% casein), the animals' growth is not increased. The present investigation was undertaken to determine whether the sulfur of isocysteine is oxidized to sulfate by the rat.

Procedure. Isocysteine hydrochloride was prepared from α-bromo-β-alanine and labeled sodium disulfide according to the method of Schöberl and Braun.[3] Five grams of α-bromo-β-alanine hydrobromide and a solution containing 325 mg of disulfide sulfur containing S^{35} equivalent to 130,000 ± 2500 c.p.m. were used in the synthesis. The sulfur in an aliquot of the solution (in 0.1 N sodium hydroxide) of sodium sulfide which was used to prepare the disulfide for the synthesis was oxidized by alkaline fusion. A small amount of carrier was added to the resultant sulfate, and the mixture was precipitated as barium sulfate. This was washed by repeated centrifuging, first with 0.001 N hydrochloric acid and then with water, and transferred as a slurry in 70% alcohol to a tared counting cup, dried, weighed, and counted[4] using a $5/\mathrm{mg/cm}^2$ end-window G-M tube and a scaler. Counting was continued for a sufficient time to insure an error of less than 2%. The sulfate later obtained from the specimens of biological material was precipitated, washed and counted in the same way as the sulfate from the original sulfide-sulfur solution except that no carrier was added. All counts were corrected for self-absorption and for decay.

The isocysteine hydrochloride as originally isolated was grossly impure and was purified by conversion to and recovery from its mercaptide. The final yield was 0.88 g (25%) and contained 24% of the initial radioactivity.

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* Present address: Hospital of the Rockefeller Institute for Medical Research, New York City.

† Present address: The Anderson Hospital for Cancer Research, Houston, Texas.

[1] Wingo, W. J., and Lewis, H. B., *J. Biol. Chem.*, 1946, **165**, 339.

[2] Sullivan, M. X., and Hess, W. C., *Pub. Health Rep.*, U.S.P.H.S. supplement No. 86, 1930.

[3] Schöberl, A., and Braun, H., *Ann. Chem.*, 1939, **542**, 274.

[4] Dziewiatkowski, D. D., *J. Biol. Chem.*, 1945, **161**, 723.

Its sulfur content was 19.4% (theory is 20.34%).

In each of 5 experiments, 100 mg of isocysteine hydrochloride (3627 c.p.m.) were given to young adult rats (140-168 g) from the McCollum colony at the Johns Hopkins University. In 3 cases the isocysteine hydrochloride was given by stomach tube; in 2 cases it was given by intraperitoneal injection. The rats were fasted for 24 hours before being given the compound; afterwards they were kept, still fasting, for 24 hours in individual metabolism cages. They were then killed with chloroform; and the radioactivities of the sulfur fractions of the urine, the feces, and the intestinal contents were determined. If the bladder contained urine at autopsy, this urine was added to the 24-hour specimen from the metabolism cage. Samples of the inorganic sulfate-sulfur, the total sulfate sulfur,[5] and the total sulfur[6] of the urine were isolated as barium sulfate. The feces and the intestinal contents were hydrolyzed with sodium hydroxide for 6 hours on a steam bath. An aliquot of each of these samples was acidified to Congo red paper with hydrochloric acid, filtered, heated for two hours longer, and extracted 3 times with ethyl ether; the total sulfate in the aqueous phase was then precipitated with barium chloride. Other aliquots of the alkaline hydrolysates were oxidized with sodium peroxide according to Bailey's method,[7] and the sulfate thus obtained from the total sulfur was precipitated as barium sulfate. The barium sulfate from these samples was isolated, weighed and counted according to the procedure already outlined.

Results. The data in Table I indicate that isocysteine hydrochloride is fairly well absorbed from the intestine of the rat within 24 hours, since in 2 of the 3 experiments in which the compound was given by stomach tube, 3% or less of the radioactivity of the dose given was recovered in the feces, and in 2 of the 3 experiments 5% or less was recovered from the intestinal contents. It is

TABLE I.
Excretion of Sulfur and Radioactivity in 24 Hours by Rats Following Administration of Isocysteine Hydrochloride Containing S^{35}.

	Urinary Sulfur						Fecal sulfur				Intestinal sulfur			
	Inorg. SO_4-S		Total SO_4-S		Total S		Total SO_4-S		Total S		Total SO_4-S		Total S	
Rat	mg	%‡	mg	%‡	mg	%‡	mg	%‡	mg	%‡	mg	%‡	mg	%‡
2*	5.2	9.5	5.5	9.9	21.4	66.9	0.5	0.16	20.0	2.7	1.6	0.45	4.8	2.7
3*	4.9	5.8	6.0	8.0	20.9	57.5	0.5	0.45	5.3	3.0	2.4	0.79	—	—
4*	5.2	6.6	6.0	8.7	21.4	54.2	1.3	1.1	11.8	14.6	1.1	0.53	8.8	5.0
5†	5.5	4.6	6.0	6.3	21.4	52.6	0.5	0.17	4.1	0.21	0.5	0.00	11.9	1.1
6†	6.3	5.2	6.6	7.5	24.4	52.4	0.5	0.09	4.4	0.80	0.4	0.22	8.5	1.3

* Given 100 mg isocysteine hydrochloride (3627 counts/minute) by stomach tube.
† Given 100 mg isocysteine hydrochloride (3627 counts/minute) intraperitoneally.
‡ % is noted as % of total activity administered.

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[5] Folin, O., *J. Biol. Chem.*, 1905-06, **1**, 131.
[6] Denis, W., *J. Biol. Chem.*, 1910-11, **8**, 401.
[7] Bailey, K., *Biochem. J.*, 1937, **31**, 1396.

of interest that a small but definite fraction of the radioactivity was recovered as sulfate and as organic sulfur from feces and intestinal contents of rats given the compound intraperitoneally. The activity of the feces in these experiments could have been due to contamination with urine despite precautions employed to prevent such contamination; this could not be true in the case of the intestinal contents, since these had never been in contact with urine.

It is apparent that, while a small portion of the sulfur of isocysteine hydrochloride given to rats either by stomach tube or by intraperitoneal injection may be oxidized to sulfate and excreted as such, a much larger fraction is still bound in organic combination when it is excreted. The rat, therefore, reacts similarly to the rabbit in that it does not readily oxidize the sulfur of isocysteine to sulfate. Quantitative differences between the two species may, however, exist. The rats employed in this investigation excreted in the urine, within 24 hours, 54 to 67% of the S^{35} administered as isocysteine by stomach tube. The rabbits employed in the earlier study[1] excreted, within 24 hours, "extra" sulfur equivalent to only 29 to 37% of the dose given by stomach tube. On the other hand, the rabbits excreted "extra" sulfur equivalent to 76 to 98% of the dose when isocysteine was administered subcutaneously; rats excreted only about 52% of the S^{35} of isocysteine which had been injected intraperitoneally. The metabolic fate of the S^{35} not found in the excreta or intestinal contents of these rats cannot be ascertained from the present data, but presumably it was still contained in the tissues.

Summary. 1. The oxidation of the sulfur of isocysteine by the rat was studied with the aid of isocysteine containing S^{35}.

2. It was found that only a small amount of S^{35} was excreted as sulfate in the urine within 24 hours after administration of the compound by stomach tube or by intraperitoneal injection. A much larger portion was excreted as organic sulfur. A very small fraction of the S^{35} was recovered in the feces and intestinal contents of the rats.

3. The results of the present study are compared with those of an earlier investigation in which rabbits served as subjects.

The authors are grateful to Dr. M. D. Kamen for the generous gift of cyclotron irradiated carbon tetrachloride from which the S^{35} employed in this investigation was isolated.

Sincere thanks are expressed to Dr. C. W. Sheppard for the loan of the scaler used in making the activity measurements.

16957

Streptomycin in the Treatment of Leptospira Carriers. Experiments with Hamsters and Dogs.*

K. T. BRUNNER AND K. F. MEYER.

From the George Williams Hooper Foundation, University of California, San Francisco.

The carrier problem dominates the control of canine leptospirosis. Dogs remain urinary shedders for from 2 to 6 months after recovery. With the introduction of chemotherapy, the risk of creating carriers and thus spreading leptospirae is greatly increased.

Penicillin and streptomycin have been found to inhibit growth of leptospirae *in vitro* and to be effective against experimental leptospirosis in guinea pigs, mice and hamsters. Shih Lu Chang[1] observed that *Leptospira icterohaemorrhagiae* disappeared from the blood of guinea pigs 3 to 5 days after 2 daily injections of aqueous solution of penicillin in total daily doses of 800 units. A

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* Aided by a grant from the National Canine Research Foundation.

[1] Chang, S. L., *J. Clin. Invest.*, 1946, **25**, 752.

serum concentration of more than 0.2 unit per cc was maintained. However, the leptospirae persisted in the liver even when the dose was increased to 3,000 units daily and continued for 6 to 8 days after the blood tests had become negative. Wylie and Vincent[2] found that unless penicillin was given to the guinea pigs 24 hours after inoculation of *L. icterohaemorrhagiae,* the guinea pigs died. These authors stated that renal damage might have made the leptospirae inaccessible to the antibiotic.

Petersen and Schmidt[3] demonstrated leptospirae in the kidneys of a guinea pig 3 months after inoculation with *L. icterohaemorrhagiae* and penicillin treatment. In experiments reported by Brunner,[4] hamsters were proven renal carriers 26 days after inoculation with *L. canicola* and penicillin treatment. In discussing these results it was emphasized that although penicillin treatment may save the life of a hamster or a dog, it may favor the development of a carrier state.

Heilman and Herrell[5,6] successfully treated hamsters infected with *L. icterohaemorrhagiae* with streptomycin. Treatment was started 17 hours after inoculation, and no leptospirae were found in the livers and kidneys of the treated animals after 28 and 33 days. In hamsters infected with *L. canicola* and treated with streptomycin, Brunner failed to demonstrate the organisms in the kidneys by culture and darkfield examination.

It would appear from the foregoing that penicillin is only effective in leptospiremia, while streptomycin either prevents the leptospirae from reaching the renal tubules or is able to destroy them there. The important question of whether leptospirae multiplying in the renal tubules of chronic carriers could be reached and destroyed by streptomycin had not been answered by these studies. It is the purpose of this paper to record experiments in this direction.

Experimental work. Series 1. Forty hamsters, 4 or 5 weeks old, were each injected intraperitoneally with 0.25 cc of a virulent *Leptospira canicola* culture (120,000 organisms per cu mm). On the 60th hour after injection each hamster was given intramuscularly 500 mg of penicillin in oil and wax, and a second dose of 250 mg 12 hours later. Four hamsters died of leptospirosis after 12 to 15 days. Thirty-two days after inoculation, 9 of the 36 surviving hamsters were killed, and kidney material was examined in the dark field and cultured. All the 9 cultures were positive for leptospirae, and in 7 cases could the organisms readily be demonstrated by darkfield illumination in the fresh specimens of kidney.

Having established that the hamsters were carriers, 18 of the series were treated on 3 successive days with 5 mg of streptomycin each intramuscularly, given in oil and wax (0.1 cc volume), and 9 hamsters were left untreated as further controls. The 18 treated hamsters and the 9 untreated controls were killed on the 2nd day after the last streptomycin treatment, and kidney material was examined microscopically in the dark field and cultured. All the 18 cultures of kidney material of the treated animals were negative for leptospirae and remained sterile, whereas the cultures of the 9 controls were all positive, and in 8 the organisms could be demonstrated on darkfield examination.

In summary, then, of 36 hamsters inoculated with a virulent culture of *L. canicola* and treated with penicillin, 18 chosen at random and killed 32 to 37 days after inoculation were renal carriers, while the remaining 18 treated on 3 successive days with streptomycin were proved to have sterile kidneys 2 days after therapy had been instituted on the 33rd day after infection.

Series 2. Five dogs (about 4 months old) were infected intraperitoneally with a virulent culture of *Leptospira canicola* (about 50 million organisms per kilo body weight). Eighteen days after inoculation large numbers of leptospirae were found in the urine of all the dogs. (One dog died during the 5th week; the findings at autopsy were typical of leptospirosis.) The urine of one of the carriers was again examined 4 weeks after inoculation and found to be heavily contaminated with leptospirae. The dog (8.5 kg) was then treated with streptomycin, 700 mg on the 1st day and 500 mg on the 5 following days, given intramuscularly in oil and wax. One day after the last treatment, results of urinalysis for leptospira were negative; the same day leptospirae were found in the urine of the 3 remaining carriers. The pH values of the urine were found to fluctuate between 6 and 8; living leptospirae were not found at pH values below 7 (the dead organisms could still easily be demonstrated in the dark field). Care was taken to obtain neutral or alkaline samples from all the dogs.

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[2] Wylie, J. A. H., and Vincent, E., *J. Path. and Bact.,* 1947, **59**, 247.

[3] Petersen, B. C., and Schmidt, R. M., *Acta path. et microbiol. Scandinav.,* 1945, **22**, 462.

[4] Brunner, K. T., *California Veterinarian,* 1948, **1**, 18.

[5] Heilman, F. R., *Proc. Staff Meet., Mayo Clin.,* 1945, **20**, 169.

[6] Heilman, F. R., and Herrell, W. E., *Proc. Staff Meet., Mayo Clin.,* 1944, **19**, 89.

The 3 untreated carrier dogs were then treated with streptomycin 3 days after urine had again been examined and organisms found (5 weeks after inoculation of the culture). The dose was 40 mg per kilo body weight for 4 days, given intramuscularly in oil and wax. On the 5th day after the beginning of treatment, organisms were not found in the urine (4 dogs). Darkfield examinations were made and urine was injected intraperitoneally into young hamsters.

Urine from the 4 treated dogs was again examined 11 days later and organisms were not found. The 4 dogs were then sacrificed and the kidneys examined, kidney material cultured and injected intraperitoneally into young hamsters. Results of these examinations were negative.

Series 3. Four dogs (4 months old) were given intraperitoneal injections, 2 with a virulent culture of *L. canicola* (about 120 million organisms per kilo body weight) and 2 with virulent cultures of *L. icterohaemorrhagiae* (about 30 million organisms per kilo body weight). One dog infected with *L. canicola* died on the 6th day of leptospirosis. Urine of the dogs was examined microscopically in the dark field 14 days after inoculation and found to contain large numbers of leptospirae. A second urine examination was made 9 days later, and results were again strongly positive. On the same day, streptomycin treatment of 2 dogs was started; 1 dog infected with *L. icterohaemorrhagiae* was left untreated. The daily dose used was 40 mg per kilo body weight given intramuscularly in oil and wax on 3 successive days. Two days after the last treatment urine from all 3 dogs was examined, and organisms were found only in the urine of the control dog. The 2 treated dogs were sacrificed, kidney material cultured and examined in the dark field with negative results.

Conclusions. The data here presented prove that chronic renal infections with leptospira in hamsters and dogs may be successfully cured with streptomycin. Although penicillin, as well as streptomycin, effectively influences the leptospiremia in experimental infections in guinea pigs, mice and hamsters, only streptomycin is capable of destroying the organisms found in the convoluted tubules.

Canine leptospirosis is usually treated after the parasite has already disappeared from the blood. Obviously, penicillin is of little value. Streptomycin in oil and wax in a daily dose of 40 mg per kilo given intramuscularly should be used in the treatment of acute and chronic canine leptospirosis. In fact, this antibiotic may be employed in the prevention of this destructive disease in dogs. It is recommended that the urine of all dogs intended for breeding be examined for leptospira, and when infection is found, the dogs should be treated for 3 to 5 days with streptomycin a few days before the act of breeding.

16958

Effect of Pteroylglutamic Acid on Weight and "Alkaline" Phosphatase of Kidneys of the Mouse.

Gilberto G. Villela and Maria Isabel Mello.
From the Biochemical Laboratory, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil.

Kidney injury in guinea pigs and rabbits treated with pterolyglutamic acid (PGA) was first reported by Harned and coworkers, although PGA when given orally to rats was not harmful as judged by the normal diuretic test of Lipschitz.[1] However, these authors found a frequent precipitation of the PGA in the renal *tubuli* of the damaged kidneys. No other reports on kidney injury produced by PGA have been published so far.[2]

In the course of a toxicity study of PGA, one of us was able to find an enlargement of the kidneys in all the mice injected with PGA, even with doses far below the toxic level.[3] It has been observed that the weight of the kidneys increases markedly probably due to the precipitation of the insoluble PGA in the renal *tubuli,* which is able to induce some modifications on the enzyme content of the tissue. It was therefore of interest to investigate whether the phosphatase of the kidney was affected by injected PGA. Our first results showed that the "alkaline" phosphatase decreases even when small doses of PGA are administered subcutaneously (0.5 mg/kg). The increase of weight seems not to be due to an apparent retention of water but probably to a lowering of the excreted solids through the urine. In the present paper, the changes of the weight and of the "alkaline" phosphatase content of the kidneys after administration of low and high doses of PGA will be reported.

Material and methods. Male white mice of a homogeneous stock were used throughout this study. The animals were fasted 16 hours before autopsy and killed by exsanguination. The kidneys were immediately removed, weighed and homogenized in a mortar with distilled water. The kidneys homogenate was diluted with 40 ml of distilled water per g of tissue, according to Kochakian and Fox.[4] The samples were filtered to remove connective tissue and a final 10-fold dilution was made. King & Armstrong's method using disodium phenylphosphate as a substrate was used with slight modifications.[5] Blank determinations were run simultaneously, the incubation period being omitted. The results were expressed in units of "alkaline" phosphatase activity, one unit being equivalent to 1 mg of phenol liberated by hydrolysis. The disodium phenylphosphate solution was prepared each 2 days and stored in the ice box. In our experience older solutions give higher blanks and erratic values.

The age of the mice being a factor influencing the phosphatase content of the kidney, as showed by Kochakian and Fox, mice were selected having the same limit of age and weight and submitted to a standard diet. Adult mice weighing 20 to 22 g (65 to 90 days old) were used. Normal kidney weight and "alkaline" phosphatase values from 54 well fed mice are summarized in Table I.

Different groups of mice were injected subcutaneously with graded doses of PGA and animals killed 24 hours after the injection. The LD_{50} for this drug given intraperitoneally was found to be 600 mg/kg.[1] In our experience the dose of 375 mg/kg killed 50% of the animals when injected subcutaneously. Therefore, we used doses far below this limit to avoid acute intoxication. No death occurred in all the mice studied except for

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[1] Harned, B. K., Cunningham, R. W., Smith, H. D., and Clark, M. C., *New York Acad. Sci.,* 1946, **48**, 289.

[2] Jukes, T. H., and Stokstad, E. L. R., *Physiol. Rev.,* 1948, **28**, 51.

[3] Villela, G. G., *Arch. Biochem.,* 1947, **15**, 157.

[4] Kochakian, C. D., and Fox, P., *J. Biol. Chem.,* 1944, **153**, 669.

[5] King, E. J., Micro-Analysis in Medical Biochemistry, London, 1946, J. and A. Churchill, Ltd., p. 57.

TABLE I.
Average Values for Normal Mice and Mice Injected with PGA.

No. of mice	PGA mg/kg	Wt of kidneys, mg	σ	S.E.	Alkaline phosphatase in units					
					Total		S.E.	per g		S.E.
31	0.5-5.0	292	14.3	3.97	104	21.5	5.96	367	75.9	2.10
57	25-500	416	11.2	1.67	61.5	17.3	2.58	146	14.5	2.16
54	Controls	269	29.4	13.36	127	21.3	9.68	469	19.4	8.81

σ = standard deviation = $\sqrt{\dfrac{\Sigma(x - m)^2}{n - 1}}$ S.E. = standard error = $\dfrac{\sigma}{\sqrt{n}}$

those few injected with the dose up to 375 mg/kg.

Since a single dose of 0.5 mg/kg has only a slight effect, we tried repeated doses to know if a cumulative effect could be obtained. Five mice weighing 20-21 g were injected daily with 0.5 mg/kg until a total amount of 5 mg/kg was attained. The average weight of the kidneys was 331 mg and the "alkaline" phosphatase values were respectively 100 and 312 units for the total kidneys and per g of tissue. These results show that repeated doses of PGA produce a cumulative effect probably due to the slow rate of PGA excretion through the kidneys. The increase of weight of the kidneys is not related to the water content since the average mean values for the normal and injected mice were not significant. In one experiment 20 normal mice were used and 20 others were injected with 0.5 mg/kg to 25 mg/kg; the results for the first group showed 76.3 ± 0.3% of water and for the second group 75.4 ± 0.8%.

The inspection of the Table I demonstrates the effect of PGA on the weight and "alkaline" phosphatase of the kidneys. The difference between the means of the injected and non-injected animals is higher than twice the standard error of the mean for all groups analysed. These results are therefore statistically significant.[6] Even if the group injected with small doses is considered a significant value is obtained.

Summary. PGA when administered subcutaneously to normal mice in a single injection, produces after 24 hr an effective increase of the total weight and a decrease of the "alkaline" phosphatase of the kidneys. Normal values based on 54 mice are reported and compared with those obtained from 88 mice injected with graded doses of PGA (0.5 mg/kg to 500 mg/kg of body weight). The "alkaline" phosphatase was determined by a slight modification of the colorimetric method of King and Armstrong using disodium phenylphosphate as a substrate. The retention and precipitation of PGA in the renal *tubuli*, could be responsible for the increased weight and decreased phosphatase activity of the kidneys. Further experiments to determine the mechanism of action of PGA on kidney phosphatase are in progress and will be reported in the near future.

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[6] Fisher, R. A., Statistical Methods for Research Workers, London, 1934, Oliver and Boyd, p. 112.

16959

Benzene-Inactivated Rabies Vaccine.

Karl Habel, J. Frederick Bell, and John T. Wright.
From the Laboratory of Infectious Diseases and the Laboratory of Biologics Control, Microbiological Institute, National Institutes of Health, Bethesda, Md.

As a part of a program aimed at purification of rabies vaccine, an earlier report[1] was made on the benzene and ether extraction of ultraviolet inactivated rabies vaccine.

The technic described in our earlier paper involved the extraction of infected, dry brain tissue containing virus inactivated by ultraviolet light. Irradiation was used for inactivation because it was known to yield vaccines of high antigenic potency, and because cold benzene and ether did not inactivate all of the rabies virus. The present paper describes a method for inactivation of rabies virus by benzene during the course of extraction of lipids with that solvent.

Materials and Methods. The procedure of inactivating rabies virus by benzene differs only slightly from the method described for extraction of brain lipids.[1] A 20% infected brain suspension in distilled water is rapidly dried from a frozen state. To the dry powder is added sufficient benzene to equal 2 times the original volume. The suspension is vigorously agitated to break up clumps of tissue, poured into a screw-top bottle, and placed in a water bath at the desired temperature. After the appropriate period of time (see below) the benzene is removed by filtration through a sintered glass filter of "M" porosity by suction. The suction is then turned off and clean benzene is added to the residue on the filter. Resuspension is accomplished by stirring with a sterile applicator stick, and again suction is applied to remove the benzene. It has been established that the volume of benzene used for the first extraction is more than sufficient to dissolve the soluble lipids in the brain tissue. The first extraction is done, therefore, to dissolve lipids. The second extraction, of short duration, is done to flush out the benzene which contains lipids.

Following the extractions with benzene, the residue on the filter is resuspended in one volume of ether. The ether is permitted to filter without suction. A second resuspension in ether is done and this time the ether is removed by suction. The purpose of the ether extraction is twofold: to remove lipids—especially phosphatids which may not be soluble in benzene, and to flush out benzene not removed by filtration.

The small amount of ether which remains is removed by placing the entire filter in a vacuum chamber for a period of about 30 minutes. The dry powder is then added to a volume of distilled water equivalent to the original volume. Mixing and hydration are accomplished by churning in a Waring blender (a drop of caprylic alcohol is used to reduce the foam thus formed).

Antigenic potency of the vaccines is determined by the mouse protection test of Habel and Wright.[2]

The Temperature and Time Necessary for Inactivation of Rabies Virus (P M strain 980). The principle of using higher temperatures for the inactivation of rabies virus in benzene was derived from the early experiments in which the virus was treated with benzene at 5°C. It was observed at that temperature that the titer of live virus was markedly decreased, but the antigenic potency was not adversely affected. Subsequently, the vaccine was extracted at 37°C and no decrease in antigenicity was observed, but the titer of live virus was more markedly diminished.

It was subsequently found that 24 hours at 37°C would usually result in inactivation of the virus but occasionally viable virus could

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[1] Wright, John T., Bell, J. Frederick, and Habel, Karl, *Science*, 1948, **108**, 118.

[2] Habel, Karl, and Wright, John T., *Public Health Rep.*, 1948, **63**, 44.

TABLE I.

Lot No.	Animal species and source of vaccine	Benzene inactivated Temp., °C	Benzene inactivated Time, hr	Benzene inactivated LD_{50} prot., × 1000	Ultraviolet inactivated, LD_{50} prot., × 1000
1	Mouse	37	144	>417	289
2	”	37	72	>289	246
3	Rabbit	37	96	1.2	1.2
4	”	37	96	0.25	0.23
5	Mouse	56	8	35	35
6	Rabbit	56	8	1.7	2.2
7	”	56	9½	10	2.8
8	”	56	12	1.2	1.4

be detected. An obvious reason for this variability in inactivation seemed to be that some clumps of dry tissue were not broken up in benzene when simply shaken in that solvent. Subsequent extractions for 24 hours at 37°C using more finely divided tissues consistently yielded non-viable antigens.

For routine inactivation of the virus, however, it is desirable to submit the tissues to more than the bare minimum of exposure usually necessary for such inactivation, as long as antigenicity is not lost by this over-exposure. There has been no indication that over-exposure to benzene at 37°C results in any loss of antigenicity, and there is direct evidence that exposure for as long as 5 days at that temperature is not harmful.

Because benzene at 37°C was not inimical to the vaccine, even on prolonged exposure, it seemed probable that a higher temperature might be used for extraction. The following data compare the potencies of vaccines prepared from one lot of rabies infected brain emulsion exposed to temperatures of 37°, 45°, and 56°C for 72 hours during benzene extraction. All 3 vaccines were negative when tested for viable virus.

37°C	for	72	hrs	LD_{50} protection	>195,000
45°C	”	”	”	” ”	>195,000
56°C	”	”	”	” ”	11,400

It is apparent that 56°C for 72 hours caused a marked reduction in antigenicity, but did not destroy it completely.

The next step was to determine the minimum time necessary for rendering all the virus non-viable at 56°C. It was found that virus thus exposed for 9 hours was inactivated, but that at 5 hours sufficient live virus remained to infect one out of 6 mice when a 10% whole brain suspension was injected intracerebrally. One hour at 56°C was sufficient to effect a ten-fold drop in titer.

The effect of prolonged heating at 56°C in benzene upon the antigenicity of the virus is exemplified below:

56°C	12 hr	LD_{50} protection	11,300
”	24 ”	” ”	27,600
”	48 ”	” ”	<4
”	70 ”	” ”	<4

The vaccine extracted for 24 hours appears to have greater antigenicity than that heated for only 12 hours, but this difference is not significant. However, at some time between 24 hours and 48 hours the potency of the vaccine is almost completely destroyed.

From these data a procedure has been derived for the routine preparation of vaccines which accomplishes the two primary objectives of high antigenicity and non-viability. In ordinary use the virus has been exposed to benzene at 56°C for 12 hours. This treatment has resulted in uniformly high potency of various lots of vaccine, and has consistently yielded non-infectious vaccines.

The Lipid Content of Benzene-Treated Vaccines. In the previous report it was stated that benzene-ether extraction at 5°C effected a removal of 41% of the total dry weight of brain tissue. This statement was in error since subsequent studies have established by extraction of brain tissue with hot alcohol and ether that the lipid content of rabbit brain and of mouse brain are 50% and 40% respectively, of the total dry weight. After extraction with benzene and ether the residual lipids constitute about 25% of the dry weight of both rabbit and mouse brain tissue.

Comparative Antigenicities of Benzene and Ultraviolet Inactivated Vaccines. The relative antigenicities of benzene inactivated as compared with ultraviolet light inactivated vaccines, each made from the same lot of virus, are listed in Table I.

In most instances, when there were differences, the benzene inactivated vaccine showed the higher potency.

In addition to these comparisons showing benzene inactivated vaccines were as good as the ultraviolet inactivated vaccines, more than 20 other lots have been inactivated with benzene alone and have consistently produced good vaccines.

Discussion and Summary. The use of benzene-ether extraction as a preliminary step in eventual purification of rabies vaccines has been made a more practical procedure by the demonstration that inactivation of the virus by heating in benzene results in a highly potent vaccine. Thus the necessity for preliminary ultraviolet irradiation has been eliminated.

16960

Rabies Vaccine Freed of the Factor Causing Allergic Encephalitis.

J. Frederick Bell, John T. Wright, and Karl Habel.
From the Laboratory of Infectious Diseases and Biologics Control Laboratory, Microbiological Institute, National Institutes of Health, Bethesda, Md.

The preliminary phases of a program for the purification of rabies vaccine have been reported in earlier papers. These reports dealt with the problems of removal of lipids from brain tissue vaccines,[1] and the inactivation of rabies virus by heating the dried tissue in a lipid solvent (benzene).[2] Earlier attempts at the purification of rabies antigen by the usual chemical and physical fractionation procedures had met with failure. Brain tissue is rich in lipids and it was thought that these might have the effect of stabilizing the suspension of other constituents including virus, thus inhibiting sharp separation. The first step in separation of antigen, therefore, was the removal of lipids. For this purpose benzene and ether were used, and it was found that vaccines thus treated did not lose antigenicity. A corollary finding was the observation that the rabies virus could be rendered non-infectious by heating during the course of benzene extraction, thus obviating the necessity for preliminary inactivation by ultraviolet light or other agents.

Postvaccinal (rabies vaccine) encephalomyelitis in man, and its apparent counterpart, experimental allergic encephalitis in lower animals, are conditions which occur when brain tissue is injected parenterally.[3-7] The encephalomyelitis may be manifest as a paralysis of the Landry type. Pathognomonic lesions are found in the central nervous system.

A consideration of the etiology of this postvaccinal encephalomyelitis had led to the hope that removal of the lipid components of brain tissue might also eliminate the material which was responsible for the paralysis.[8] However, it was found that benzene-ether extracted vaccine was essentially unaltered in its capability of producing paralysis in guinea pigs.

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[6] Morgan, I. M., *J. Exp. Med.*, 1947, **85**, 131.

[7] Wolf, A., Kabat, E. A., and Bezer, A. E., *J. Neuropath. and Exp. Neurol.*, 1947, **6**, 333.

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The present report will describe the further treatment of rabies vaccines which has resulted in antigens of high potency, yet freed of the factor which causes allergic encephalitis in experimental animals.

Materials and Methods. In each experiment, one lot of vaccine, or a pool of several lots was used, and comparisons of potency were made between various fractions of the one lot or pool. In every instance the various fractions were brought back to the volume of the original vaccine suspension before being compared. In some of the early experiments the potencies of the vaccines used were so great that no end-point of protection in mice was obtained, and therefore no comparisons of the potencies could be made. In later experiments, an attempt was made to select lots of vaccine which would protect mice when challenged with 100 to 10,000 LD_{50}, which figures are within the usual range of the end points of the mouse protection test. Both mouse and rabbit brain vaccines were used. In all of the tests for the factor causing allergic encephalitis, only rabbit brain vaccines were employed.

In order to elicit the signs and lesions of allergic encephalitis, guinea pigs were injected in the nuchal area with vaccines made up in mineral oil containing killed *M. tuberculosis* and Arlacel-A according to the method of Freund.[3,9] One injection of one ml of the adjuvant mixture was made subcutaneously in the nuchal area of the guinea pigs, and early signs of encephalitis usually ensued about 12 to 14 days later. The animals were observed for about 7 weeks after injection. Characteristic lesions of allergic encephalomyelitis sometimes occur in guinea pigs which remain asymptomatic. Therefore, random samplings, or in some cases, all of the guinea pigs in groups without signs of encephalomyelitis were killed at the termination of the experiments, and the brains and cords were examined for microscopic lesions.*

Calcium Acetate as a Precipitant for Virus Antigen. In the course of some experiments on the elution of live rabies virus from tissue it was observed that calcium acetate greatly reduced the titer of virus in the supernatant liquor of a centrifuged suspension of rabies brain. That this effect was not caused by killing the virus was proved by resuspending the sediment (in molar sodium chloride solution) and again centrifuging the suspension. The titer of virus thus obtained in this second supernatant fluid was as high as was ordinarily expected in the original. Further investigation revealed that the virus treated with calcium acetate, and apparently precipitated by it, could not be resuspended in water. Non-viable virus antigen likewise was precipitated by calcium acetate and remained insoluble in water. This precipitated antigen retained its antigenic potency.

In the first experiment rabies-infected brain was homogenized in a 1% solution of calcium acetate. The mixture was centrifuged and the titer of virus in the supernatant fluid was found to be low. Further experiments revealed that concentrations as low as M/100 (0.158%) of calcium acetate could be used with maximal effect. In these experiments the brain tissue vaccines were suspended in various concentrations of calcium acetate and filtered. The filtrates were then used as antigens in the mouse potency test.

It is evident here that calcium acetate in concentrations of M/100 or greater prevented passage of antigen through the filter.

The insolubility in water of virus antigen precipitated by calcium acetate is shown in Table II. In these experiments, vaccines which had been extracted by benzene and ether were suspended in water ("original"). Sufficient calcium acetate solution was added to the suspensions to make a final concentration of M/10 and the suspensions were centrifuged. (The supernatant fluid is referred to as "calcium acetate wash"). After decanting the supernatant fluid, the sediment was resuspended in a volume of distilled water equal to the first supernatant fluid, and the mixture was again centrifuged. (The supernatant

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[9] Freund, J., Thomson, K. J., *et al.*, *J. Immun.*, 1948, **60**, 383.

* The pathological studies were done by Dr. James Peers.

TABLE I.
Antigenic Potency of Filtrates of Vaccines in Various Concentrations of Calcium Acetate in H_2O, Expressed in LD_{50} Protection.

Exp. No.	M/10	M/100	M/1000	No Ca Ac
A 168	<22	<22	>220,000	220,000
A 179	<36	<36	1,500	20,000

TABLE II.
Antigenic Potency of Vaccine Fractions in LD_{50} Protection.

Exp. No.	Original	Cal. acetate wash	Water wash	Residue
A 187	69,000	420*	<42	11,000
A 188	2,900	<29	<29	360
A 195	1,200	<17	<17	1,100

* M/100 calcium acetate was used instead of M/10 and insufficient time for reaction was allowed.

TABLE III.
Comparative Potencies of Original and Washed Vaccines (LD_{50} Protection).

Lot	Original	Washed
A 142	>381,000	>381,000
A 152	470	1,200
A 158	> 66,100	17,800
A 163	> 32,400	> 32,400
A 177	>513,000	16.300

fluid is labeled "Water Wash"). The sediment was resuspended in distilled water to the original volume ("Residue").

The comparative antigenic potencies of other benzene-ether extracted vaccines and their washed calcium acetate residues are presented in Table III.

It has been observed that the most effective way of adding calcium acetate to the vaccine is by the addition of a solution of the chemical to a suspension of the vaccine. (Other methods such as the addition of the calcium acetate to the vaccine before drying and extracting with benzene and ether, adding the dried extracted powder to a solution of calcium acetate, or adding the chemical to a vaccine resuspended after extraction were tried. The last procedure was satisfactory but less consistent in results.)

It will be noted in the experiments recorded in Table II that the potencies of the residues are less than those of the control vaccines. While variations of less than three-fold may be within the limits of error of the mouse potency test, some of the apparent loss of potency seems to be the result of the poor dispersion of the precipitated antigen when resuspended. This belief is substantiated by the decrease in antigenicity caused by the addition of calcium acetate directly to vaccine as compared to the same whole emulsion vaccine without calcium acetate. (See Table IV.)

This effect could be interpreted as an actual diminution of antigen, but the lack of toxicity of calcium acetate to live virus, and the relative constancy of the effect without regard to time of exposure or concentration of the salt have led us to believe that the difference is caused by poorer dispersion of the precipitated antigen.

The Nitrogen and Calcium Contents of Washed Vaccine. Repeated analyses of vaccine before and after washing with M/10 calcium acetate followed by water have revealed that the first wash (*i.e.*, with calcium acetate) removes about 45% of the total nitrogen, and the subsequent washing with water removes about 5%. The residual washed vaccine then contains about 50% of the total nitrogen of the original vaccine. A small portion of the calcium added to the vaccine is retained by the insoluble tissue components. Most of this is removed by

TABLE IV.
Effect of M/10 Calcium Acetate on Antigenic Potency* of Crude Rabies Vaccine.

Vaccine	Without calcium acetate	With calcium acetate
A 183	>355,000	60,300
A 188 K	10,300	2,900
A 188 L	2,900	1,100

* Potency is expressed in LD_{50} protection.

TABLE V.
Pathogenic and Antigenic Potencies (LD_{50} Protection) of Crude and Washed Vaccines.

			Allergic encephalitis	Antigenic potency
A188	Unextracted	Original vacine	9/10	1,100
		Cal. acetate wash	1/8	<29
		Washed vaccine	2/9	130
	Extracted	Original vaccine	4/10	2,900
		Cal. acetate wash	9/10	<29
		Washed vaccine	0/9 *	360
A195	Unextracted	Original vaccine	5/8	1,400
		Cal. acetate wash	5/10	<17
		Washed vaccine	7/10	1,200
	Extracted	Original vaccine	7/10	1,200
		Cal. acetate wash	8/10	<17
		Washed vaccine	0/10†	1,000

The denominator indicates the number of animals injected. The numerator indicates those specifically affected.

* Five animals were killed and the brains and cords were submitted to histological study. None showed lesions of allergic encephalitis.

† All of the animals in this series were killed and the brains and cords were found free of the lesions of allergic encephalitis.

washing with water. The final calcium content of the washed vaccine (10% vaccine) is then about 0.005%.

Extraction of the Factor Causing Allergic Encephalomyelitis. When it was discovered that much water soluble material could be removed from the vaccine without loss of antigen by the use of the calcium acetate it seemed worth while to test the various fractions thus obtained for the encephalitic effect. Consequently, a portion of a lot of vaccine, after washing with calcium acetate, was then treated with molar saline in an attempt to elute antigen from the sediment. The original lot of vaccine, the calcium acetate wash liquor, the molar saline wash liquor, and the final residue were each mixed with adjuvant and injected into guinea pigs. The results were as follows:

Original vaccine (Lot A169)	2 of 8	guinea	pigs	paralyzed
Calcium acetate wash	7 of 10	”	”	”
Saline wash	4 of 10	”	”	”
Residue	None of 9	”	”	”

It was obvious then that one washing with calcium acetate was insufficient to remove all the factor. However, it was equally evident that the factor was soluble in the calcium acetate solution. That guinea pigs injected with the second wash liquor were also affected could readily be explained by the fact that not all of the calcium acetate solution could be removed from the residue by drainage. That this encephalitic factor can be completely removed by a second washing with water is demonstrated by the experiments reported in Table V.

In these experiments the effects of the washing procedures on antigenicity as well as the encephalitic factor of the vaccines were tested. Both benzene-ether extracted and unextracted irradiated vaccines were subjected to the calcium acetate treatment. The extracted and unextracted vaccines of each lot were suspended in M/10 calcium acetate solution and samples were removed for testing ("Original"). The remainder was centrifuged and the supernatant fluid ("calcium acetate wash") replaced by an equal volume of distilled water. The sediment was resuspended in water and again centrifuged. After decanting and measuring the supernatant fluid, the sediment was suspended in the same quantity of M/1 saline. Antigenic potency as recorded in Table V in the row marked "washed vaccine" was determined for this resuspended sediment. However, for the detection of the factor causing allergic encephalitis in the washed vaccines only the liquid phase of the mixture was used.

The data indicate that the factor which causes encephalomyelitis was present in the original vaccines, both extracted and irra-

TABLE VI.
Absence of the Encephalitic Factor from Washed Vaccines (Whole Washed Sediments).

	Vaccines	Encephalitis	Antigenicity (LD_{50} protection)
A 200	Original vaccine	8/10	4,080
	Washed vaccine	0/10	1,000
A 201	Original vaccine	3/5	340
	Washed vaccine	0/5	120

diated, and that it was soluble in the calcium acetate washes. After washing once with calcium acetate solution, and once with distilled water, no more of the factor could be demonstrated in the vaccines which had previously been extracted with benzene and ether. However, the method does not appear to be effective with vaccine which has not been previously extracted.

Further evidence that the factor has been completely removed from washed vaccine is presented in Table VI where the whole washed sediment was tested.

Discussion. By a process of extraction with calcium acetate solution, rabies vaccine may be freed of the factor which causes allergic encephalitis. Absence of the encephalitic factor from the washed vaccine was first indicated by the fact that it was not present in the liquid phase of the final washed vaccine. Further proof was obtained by injecting the whole washed vaccine into guinea pigs without producing paralysis.

In the experiments recorded here calcium acetate has been used because it has been found to give satisfactory results. No doubt other salts of calcium, and salts of other alkaline earth elements may be substituted. In fact, calcium chloride has been found to react very similarly.

Whether the allergic encephalomyelitis as produced in guinea pigs is identical with that occasionally seen in man following rabies vaccination is not known, but the signs and lesions, together with the common factor of development of the syndromes following the injection of brain tissue suggest that the conditions are actually similar.

The results of our experiments have been reported here because of their possible application to the production of safe rabies vaccine for human use. It is presumed that washed rabies vaccines which no longer produce allergic encephalitis in experimental animals will likewise not cause post vaccinal paralysis in man. Further purification of vaccine for human use is desirable, and the problem is being studied.

Summary. A method has been presented for the removal from rabies vaccine of the factor which causes allergic encephalomyelitis. This factor is not removed by extraction with benzene and ether, but preliminary treatment of the vaccine with these solvents facilitates separation by subsequent treatment. The presence of calcium acetate prevents the loss of antigen when the vaccine is washed. It does not prevent removal of the encephalitic factor which appears to be water soluble. About one-half of the total nitrogen of the vaccine is removed by this washing process. The technic may be briefly summarized as follows:

1. A suspension of infected brain in water is dried from the frozen state.
2. The dried brain is extracted with benzene followed by ether (Live virus may be killed in this stage by heating in benzene).
3. After removal of the ether the dried brain is suspended in distilled water.
4. Sufficient solution of calcium acetate is added to make final concentration of M/10 calcium acetate, and the suspension is permitted to stand in the cold for an hour or two.
5. The calcium acetate solution is removed by centrifugation or filtration and the sediment is resuspended in distilled water to the original volume with agitation (clumps of sediment must be broken up).
6. The distilled water is removed by centrifugation or filtration.
7. The sediment is resuspended in distilled water or saline and homogenized. This is the washed vaccine.

16961

Antagonism Between Effects of P-Aminosalicylic Acid and Salicylic Acid on Growth of *M. tuberculosis*.

G. Ivánovics. (Introduced by G. Gomori.)

From the Institute of General Pathology and Bacteriology, University of Szeged, Hungary.

It has been reported[1,2] that the bacteriostatic action of PAS is not antagonized by small amounts of salicylic acid. Lehmann[3] found in some of his experiments that a combination of PAS and salicylic acid gave a moderate inhibition of oxygen uptake of tubercle bacilli, although each of these acids, added separately, increased the rate of oxygen uptake. It was expected that an antagonism between salicylic acid and P-aminosalicylic acid (PAS) can also be demonstrated in cultures of tubercle bacilli if the experimental conditions are chosen correctly.

Methods. Cultures were grown in the liquid medium described by Dubos. It contained 0.05% of Tween 80 and 0.5% of bovine albumin fraction. A freshly prepared solution of the Na salt of PAS was added to the medium and serial dilutions by a factor of 2 were made. The tubes were inoculated with 0.0001 mg (dry weight) of tubercle bacilli grown in Dubos medium, and the total volume of the cultures was made up to 5 ml with dist. water. After 16 days of incubation at 37°C, 0.5 ml of phosphate buffer pH 7.0 containing 2% formaldehyde and 0.1% Tween 80 was added to each tube and the amount of tubercle bacilli was determined by turbidimetric readings in a Leitz universal colorimeter. Using a control culture as reference, the growth rate of bacilli in the presence of antiseptics was expressed in percentages of the full growth. Each dilution of drug or combination of drugs was tested in duplicates, and turbidimetric readings were averaged.

Four strains of *M. tuberculosis var. hominis* were used in these experiments. Two of them were isolated from sputa recently (K3 and K5); the others (Nos. 7800 and 5100) have been maintained for several years in our collection.

Results. Using strain 7800, the effect of PAS alone and in combination with sodium salicylate was studied simultaneously.

The striking antagonism between Na salicylate and PAS is shown in Table I. Although a concentration of 200 μM of Na salicylate alone markedly inhibited the growth of tubercle bacilli, this amount antagonized the effect of about 30 minimal inhibiting concentrations of PAS. The tuberculostatic action of PAS is antagonized only by a considerable concentration of Na salicylate, and the molar ratio of PAS to Na salicylate in the range of minimal inhibition varied between 0.08 to 0.25, *i.e.*, one mole of PAS was antagonized by 4 to 12 moles of Na salicylate in this experiment.

In the presence of small concentrations of Na salicylate antagonism gradually diminishes, and at the highest dilution of drug tested (20 μM) only a slight effect was seen.

As the tuberculostatic action of PAS is antagonized by p-aminobenzoic acid (PAB),[4] we determined the molar ratio of PAS to this metabolite under similar experimental conditions. It was found that one mole of PAB abolishes the effect of about 4 moles of PAS. On comparing these values it is seen that the antagonistic effect of PAB is 16 to 50 times greater than that of salicylate.

In the presence of salicylate at a concentration of 620 μM the growth of strain 7800 was found to be zero on turbidimetric read-

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[1] Youmans, G. P., Raleigh, G. W., and Youmans, A. S., *J. Bact.*, 1947, **54**, 409.

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TABLE I.
Growth Rate of *M. tuberculosis var. hominis* (Strain 7800) in Percentages in the Presence of PAS and Salicylate. (Uninhibited growth = 100%.)

	PAS μM								
Salicylate, μM	50	25	12.5	6.25	3.12	1.56	0.76	0.38	0.00
200	0	18	29	32	40	40	46	57	65
80	0	0	0	9	46	61	65	68	94
40	0	0	0	0	8	35	50	70	96
20	0	0	0	0	0	11	53	72	100
0	0	0	0	0	0	0	26	67	100

ing. This complete inhibition was not antagonized by either PAB or pantothenic acid. A slight growth (about 8 to 12%) was, however, found if a certain concentration (about 2 μM of PAS), which in itself alone is fully bacteriostatic, was added to the tube.

Similar observations were made with strains 5110 and K3. The effect of salicylate on strain K5, however, was found to be very slight.

The tuberculostatic effect of PAS was not antagonized by compounds related to salicylic acid. No antagonism was found when the Na salts of benzoic, phthalic, o-aminobenzoic, o-phenolsulfonic, o-chlorobenzoic, o-nitrobenzoic, dithiosalicylic, o-sulfobenzoic, p- and m-oxybenzoic, 2, 4-dioxybenzoic and 2-oxy-4-aminosulfobenzoic acids were tested in the range of concentrations between 40 and 200 μM. m-Aminophenol and pantothenic acid were also found to be ineffective.

Discussion. The action of salicylate on bacteria is a variable one. The growth of some non-acid fast bacteria is prevented by the drug at high concentrations by inhibiting the synthesis of pantothenic acid in the cells.[5] The non-pathogenic acid fast bacteria are capable of oxidizing salicylic acid,[6] and the oxygen uptake of resting virulent tubercle bacilli is increased in the presence of this drug;[7] however, it is not metabolized[8] by the cells. Fitzgerald and Bernheim[9] concluded from their experiments that acid fast bacteria produce an adaptive enzyme in the presence of salicylate, and that the formation of this enzyme is inhibited by streptomycin. On the basis of these observations it can be assumed that both non-acid fast bacteria requiring no exogenous pantothenic acid for growth and various acid fast bacteria either contain or form adaptively an enzyme with specific affinity to salicylic acid. However, the synthesis of pantothenic acid in tubercle bacilli is not inhibited by this drug. The nature of this enzyme (or enzymes) occurring in a wide variety of bacteria is entirely obscure at present.

Salicylate antagonizes the tuberculostatic effect of PAS at concentrations so high as to be toxic by themselves. As found previously, salicylate has a twofold action on bacteria: in addition to a specific effect at high dilutions, the drug will damage the cells in high concentrations by its protein-denaturing effect.[5,10] This latter action of the drug renders the study of antagonism of the two drugs investigated more difficult; however, it seems that it is a competitive one.

Summary. The bacteriostatic effect of p-aminosalicylic acid is antagonized by high concentrations of salicylic acid. This effect of salicylate is highly specific and not shared by related compounds. Pantothenic acid does not inhibit the tuberculostatic effect of salicylate.

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[6] Bernheim, F., *J. Biol. Chem.*, 1943, **143**, 383.

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[9] Fitzgerald, R. J., and Bernheim, F., *J. Bact.*, 1948, **55**, 765.

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16962

Aureomycin in Urinary Infections Due to Gram Negative Organisms.*

Alexander M. Rutenburg and Fritz B. Schweinburg.
(Introduced by J. Fine.)

From the Kirstein Laboratory of Surgical Research, Beth Israel Hospital, and the Department of Surgery, Harvard Medical School, Boston, Mass.

Aureomycin hydrochloride ("Duomycin")† has been reported to be a non-toxic antibiotic effective against a variety of gram-positive and gram-negative bacteria, rickettsiae and several of the larger viruses.[1-4]

This report deals with the results of aureomycin therapy in urinary tract infections due to gram-negative bacilli in 10 patients, 9 of whom did not respond to sulfonamides, penicillin or streptomycin.

In vitro studies. Data on the *in vitro* sensitivity to aureomycin of bacteria commonly encountered in urinary infections were obtained prior to the clinical study.

Serial dilutions of aureomycin in sterile broth (pH 6.8) were prepared in concentrations ranging from 200 - 0.0125 μg/ml. To 0.9 ml of each dilution 0.1 ml of a diluted 24 hour growth, containing 1500-25,000 organisms of the strain to be tested, was added. A control tube containing the same number of bacteria in 1.0 ml of sterile broth was also prepared. The tubes were incubated for 24 hours and then examined for bacterial growth. Ten strains each of *Pseudomonas aeruginosa* (*B. pyocyaneus*), *Bacillus proteus vulgaris*, *Aerobacter aerogenes*, and *Escherichia coli* were tested for aureomycin sensitivity in this manner. All strains were isolated from infected urines and had undergone only 1-3 passages. The results, listed in Table I, indicate the lowest bactericidal and bacteriostatic titers of aureomycin. The bacteriocidal titer was taken to be the lowest concentration which completely inhibited macroscopically visible growth. The bacteriostatic titer was taken to be the lowest concentration which caused less growth than in the control tube.

The results, only half of which are listed, show that different strains of a given species of gram-negative bacteria have a wide range of sensitivity. Thus, the bactericidal titer of *Ps. aeruginosa* varies from >200 μg to 0.4 μg; of *B. proteus* from 50 μg to <0.0125 μg; of *A. aerogenes* from >200 μg to 0.05 μg; and of *E. coli* from 25 μg to 0.1 μg. *Ps. aeruginosa* and *B. proteus* have been reported[1,4] to require very high concentrations of aureomycin (100-250 μg/ml). We found this to be true of some strains, but not of others. The bacteriostatic titers for a given strain of *Ps. aeruginosa, B. proteus* and *A. aerogenes* were identical in most instances with the bactericidal titer; but for *E. coli* a marked difference in the two titers was the rule. The bactericidal titer of most strains of all bacteria tested varied from 1.0 μg to 0.0125 μg, indicating a high degree of sensitivity. The bactericidal titer was always the same for a given strain tested with different lots of aureomycin.

Clinical Observations. Ten consecutive patients with a variety of urinary tract infections were treated with aureomycin. Prior therapy with sulfonamides in 7, with penicillin in 6, and with streptomycin in 2 had failed to yield any therapeutic effect. In all instances the infecting bacteria had been tested and found to be resistant *in vitro* to strepto-

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* Aided by a grant from the Patrons of Research, Beth Israel Hospital.

† An antibiotic isolated from *Streptomyces aureofaciens* and supplied by Lederle Laboratories Division, American Cyanamid Co.

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TABLE I.
Sensitivity of Gram Negative Bacilli to Aureomycin *in vitro.*

Strain	No. of bacteria, × 1000	Bactericidal titer, in μg/ml	Bacteriostatic titer, in μg/ml
Ps. aeruginosa 1	20	>200	>200
,, ,, 2	18	100	100
,, ,, 3	20	50	50
,, ,, 4	15	12.5	12.5
,, ,, 5	25	3.2	3.2
,, ,, 6	25	0.8	0.8
,, ,, 7	18	0.4	0.4
B. proteus 1	2.5	50	50
,, ,, 2	1.5	25	12.5
,, ,, 3	3	6.25	6.25
,, ,, 4	4	0.05	0.05
,, ,, 5	1.5	0.025	<0.012
,, ,, 6	1.5	<0.012	<0.012
A. aerogenes 1	2	>200	>200
,, ,, 2	2.5	100	100
,, ,, 3	2	3.2	3.2
,, ,, 4	2.5	0.4	0.4
,, ,, 5	2	0.05	0.05
E. coli 1	4	25	6.25
,, ,, 2	5	6.25	3.2
,, ,, 3	6	1.0	1.0
,, ,, 4	5	0.1	0.1

mycin, penicillin, and the sulfonamides.

Four patients had univalent and 6 polyvalent infections. All patients had pyuria and the usual clinical signs and symptoms.

Aureomycin was given intramuscularly to 9 patients. The dose usually was 80 mg every 8 hours for 5-6 days, but acutely ill patients received an initial intravenous dose of 300 mg in 500 cc of saline. The tenth patient received aureomycin orally, 4 g the first day, 3 g the second day and 2 g daily thereafter, in divided doses. The duration of treatment in all cases was based on the clinical and bacteriological response. No toxic reactions were encountered. The intramuscular injections were painful. The pain was partly or wholly alleviated by dissolving the drug in 1% procaine. Table II lists the clinical diagnosis, the bacteriological findings before and after aureomycin therapy, *in vitro* sensitivity tests of the bacteria involved to various antibiotics, and the therapy given prior to aureomycin.

The results were evaluated on a clinical and a bacteriological basis. The clinical symptoms cleared up in all patients. In 3 of the 4 patients with univalent infections and in 5 of the 6 patients with polyvalent infections the urine became sterile and remained so during the 7-10 days after the drug was discontinued. In one case (No. 3), the urine was not completely sterilized, but the plate count showed a marked reduction in the number of colonies of the responsible bacillus (*A. aerogenes*). This strain, tested *in vitro* for sensitivity, was resistant to sulfathiazole, sulfadiazine, sulfamethazine, penicillin, and streptomycin. The bactericidal titer with aureomycin was higher than 200 μg. In the second case, (No. 6), one of the 2 infecting organisms, *Ps. aeruginosa,* was eliminated, while the plate count of the other, *A. aerogenes,* was markedly reduced. This latter strain was also resistant *in vitro* to the 3 sulfonamides, penicillin and streptomycin. The bactericidal titer with aureomycin was again higher than 200 μg.

Of the 20 strains of bacteria found in the 10 patients, all strains of *E. coli* (4), of *Ps. aeruginosa* (4), of *B. proteus* (2), of *Strep. viridans* (2), of *Strep. fecalis* (1), and 5 of the 7 strains of *A. aerogenes* were cleared from the urine. The two strains of *A. aerogenes* which persisted showed a high resis-

TABLE II.
The Data of Ten Patients with Urinary Infections Successfully Treated with Aureomycin.

Case No.	Diagnosis	Urine cultures before aureomycin	Growth inhibiting concentration *in vitro* of aureomycin, μg/ml	Urine cultures after aureomycin	Sensitivity tests *in vitro* with other antibiotics*	Chemotherapy prior to aureomycin	Aureomycin	
							Daily Dose (mg)	Days
1	Chronic pyelonephritis, renal insufficiency	*A. aerogenes* *E. coli* *Strep. viridans*	6.25 3.2 0.8	Sterile	All 3 strains resistant to SD, ST, SMT, P and S.	None	240	6
2	Chronic pyelonephritis, ectopic kidney	*E. coli* *B. proteus*	0.05 12.5	,,	Both strains resistant to P, S, SD, and SMT. *Proteus* also to ST. *Coli* moderately sensitive to ST	SD	240	5
3	Chronic pyelonephritis	*A. aerogenes* heavy growth	>200	*A. aerogenes* 1-4 colonies	Strain resistant to P, S, SD, ST, SMT	Penicillin Streptomycin	80	8
4	Pyelonephritis following prostatectomy	*B. pyocyaneus* *Strep. fecalis*	50	Sterile	*B. pyocyaneus* resistant to SD, ST, SMT, P, and S.	Penicillin SD	120	6
5	Same	*E. coli* *A. aerogenes* *B. pyocyaneus* *B. proteus* *Strep. viridans*	25 200 0.25 0.25	,,	*Coli, aerogenes, pyocyaneus,* and *proteus* resistant to P, SD, ST, SMT. *Coli* and *aerogenes* slightly sensitive to S. *Proteus* and *pyocyaneus* resistant to S.	Penicillin SD SMT	160	10
6	Same	*A. aerogenes* *B. pyocyaneus*	>200 1.6	*A. aerogenes* 3-6 colonies	Both strains resistant to P, S, SD, SMT. *B. pyocyaneus* resistant to ST. *A. aerogenes* sensitive to ST.	SD Penicillin	160– 240	15
7	Chronic cystitis	*E. coli* *A. aerogenes*	0.2 0.2	Sterile	Both strains resistant to P, S, SD, SMT. Moderately sensitive to ST.	Penicillin Streptomycin	60	12
8	Acute pyelonephritis and cystitis due to renal calculi	*A. aerogenes* heavy growth	0.2	,,	Strain resistant to P, S, SD, SMT, ST.	SD Penicillin	240	5
9	Acute pyelonephritis and cystitis due to renal calculi	*B. pyocyaneus* heavy growth	12.5	,,	*B. pyocyaneus* resistant to P, S, SD, SMT, ST.	SD Penicillin	240 2000†	4 3
10	Acute postoperative cystitis	*A. aerogenes* heavy growth	0.0125	,,	*A. aerogenes* resistant to P, S, SD, ST, SMT.	SMT	2000–† 4000	6

* SD—Sulfadiazine. ST—Sulfathiazole. SMT—Sulfamethazine. S—Streptomycin. P— Penicillin.
† Oral administration.

tance to the drug *in vitro,* but were nevertheless reduced in number. Strains of *Ps. aeruginosa* and *B. proteus* which are sensitive *in vitro* to aureomycin can be eliminated from urine infected with these organisms. In all patients who were cured treatment was continued for 2 or 3 days after the urine was first found to be sterile, because in one patient not listed, on whom the drug was discontinued as soon as the urine became sterile, recurrence took place within 2 days.

Summary. 1. Aureomycin is highly effective against many strains of gram-negative bacilli *in vitro,* including many which were found to be resistant to sulfonamides, penicillin, and streptomycin. The sensitivity of various strains of a given species, however, varies greatly.

2. Clinical trial in 10 patients with urinary infections which were refractory to previous treatment with other antibiotics, resulted in a clinical cure of all ten patients. Bacteriologically eight patients were cured, and 2 were markedly improved.

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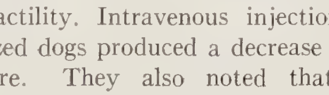
Effects of a Quaternary Amine* Capable of Blocking Functions of the Autonomic Nervous System.

F. H. LONGINO,† J. R. CHITTUM, AND K. S. GRIMSON.
From the Department of Surgery, Duke University School of Medicine, Durham, N. C.

Much interest has been directed in recent years to functions of the autonomic nervous system, and to effects of drugs which interfere with activation of adrenergic or cholinergic end organs or with cholinergic synaptic transmission in autonomic ganglia. Acheson and Moe[1] and Lyons[2] have reported effects of the tetraethylammonium ion (TEABr) in the experimental animal and in man, and have shown that it blocks cholinergic transmission at autonomic ganglia and does not prevent activation of adrenergic end organs.

2, 6 dimethyl diethyl piperidinium bromide (SC 1950), a quaternary amine (Fig. 1), has been synthesized and studied by I. C. Winter and associates.[3] They found that this drug was 7 times as potent as TEABr in blocking transmission through the superior cervical ganglion of the cat and 5 times as potent as TEABr in producing block of transmission through the ganglion of the pelvic nerve which supplies the urinary bladder of the dog. It had little spasmolytic action on excised intestine, but in anesthetized and unanesthetized dogs following a preliminary contraction it generally reduced intestinal tone and contractility. Intravenous injection in anesthetized dogs produced a decrease of blood pressure. They also noted that large doses (20-25 mg/kg) in unanesthetized animals produced a flaccid paralysis and respiratory death resembling that caused by curare.

Experiments were undertaken to investigate effects of this drug upon the circula-



2,6 dimethyl diethyl piperidinium bromide

FIG. 1.

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* Drug number SC 1950 furnished by the Research Laboratories of G. D. Searle and Co. Aided by a grant from G. D. Searle and Co.

† U. S. Public Health Service Research Fellow in Surgery.

[1] Acheson, G. H., and Moe, G. K., *J. Pharm. and Exp. Therap.*, 1946, **87**, 220.

[2] Lyons, R. H., Moe, G. K., Neligh, R. B., Hoobler, S. W., Campbell, K. N., Berry, R. L., and Rennick, B. R., *Am. J. Med. Sc.*, 1947, **213**, 315.

[3] Personal communication from Dr. I. C. Winter of the Research Laboratories of G. D. Searle and Company.

tory system and gastrointestinal tract of dogs and of man.

I. Action of SC 1950 in the dog. A. Effect on the circulatory system. Pulse rate, blood pressure, vasomotor reflexes, and response to epinephrine and neostigmine were determined in seven dogs weighing 9-16 kg. Each was anesthetized using intravenous chloralose, 0.1 g/kg. A tracheal cannula was inserted, the carotid arteries were isolated, and the vagi were divided. A left lumbar sympathectomy was performed transperitoneally. The right femoral artery was cannulated proximally and distally. The left was ligated proximally and cannulated distally. This permitted recording of 3 blood pressures using a mercury manometer connected to the proximal cannula for systemic blood pressure and 2 others connected to the distal segments for back pressure from the 2 femoral arteries. Respirations were measured by a pneumograph connected to a recording tambour.

After preparation of each animal standard tests of the circulation were employed. Carotid sinus reflexes were elicited by occlusion of both common carotid arteries using bulldog clamps applied for one minute. Peripheral vagal stimulation was accomplished using for 15 seconds a tetanizing current from a Harvard inductorium set at 7 or 8 cm. For central vagal stimulation a tetanizing current was also used for 15 seconds but the setting of the inductorium was reduced to 6 or 7 cm. Anoxia was produced by administering 7% oxygen and 93% helium for a period of 3 to 5 minutes without permitting rebreathing. Epinephrine was injected as a 1:10,000 solution through a venous cannula. Neostigmine methylsulfate, 0.5 to 1.0 mg, was also given intravenously. After preparation of the dog and performance of standard tests SC 1950 was injected intravenously in doses of 0.5 to 20 mg/kg. Tests were then repeated, contrasting results after drug with those before. Usually the drug was again administered several times using larger amounts and repeating tests before and after each injection. Three of the 7 dogs received small amounts (0.02 to 1.0 mg/kg) in repeated doses. Four received 5 to 20 mg/kg at each injection, an amount apparently necessary for constant therapeutic effects.

(1) *Effect on blood pressure and pulse.* Initial mean systolic blood pressure of anesthetized animals varied from 80 to 120 mm Hg. With 3 exceptions initial and subsequent doses of SC 1950 in amounts varying from 0.05 mg/kg to 20 mg/kg reduced pressure. The 3 exceptions occurred when blood pressure had been reduced by previous injections. Transient increases of pressure then occurred ranging from 6 to 34 mm Hg and lasting less than 2½ minutes. Other than for these three exceptions SC 1950 promptly reduced pressure. With doses ranging from 0.05 to 1.0 mg/kg reductions of blood pressure were moderate and transient, lasting 2 to 5 minutes. As dose was increased depression of blood pressure became more pronounced and duration of the period of depression lengthened. Following doses of 5 to 20 mg/kg blood pressure was reduced to ½ or 1/3 the level before injection and depression persisted for 25 to 50 minutes. A representative effect is shown in Fig. 2. After long periods of anesthesia and after several doses of SC 1950, blood pressure gradually declined and did not return to its initial level between injections.

Effect on cardiac rate also varied with amount of drug injected. Small doses (0.02 to 1.0 mg/kg) produced no change. Larger doses (5 to 20 mg/kg) caused slowing of heart rate which persisted as long as did depression of blood pressure. Before larger doses of SC 1950 pulse rates varied from 104 to 208, averaging 159. Following 5 to 20 mg/kg, pulse rates fell to from 56 to 132, averaging 93.

(2) *Effect of SC 1950 on vascular reflexes.* In the 4 animals receiving larger amounts of SC 1950 the rise of blood pressure normally occurring in response to occlusion of the carotid arteries was uniformly prevented. This response was completely abolished in each of 10 test periods following injections of 5 mg/kg or more. (Fig. 2) Stimulation of the proximal end of the divided right vagus normally produced marked increase of blood pressure and cessation of respiration (Fig. 2).

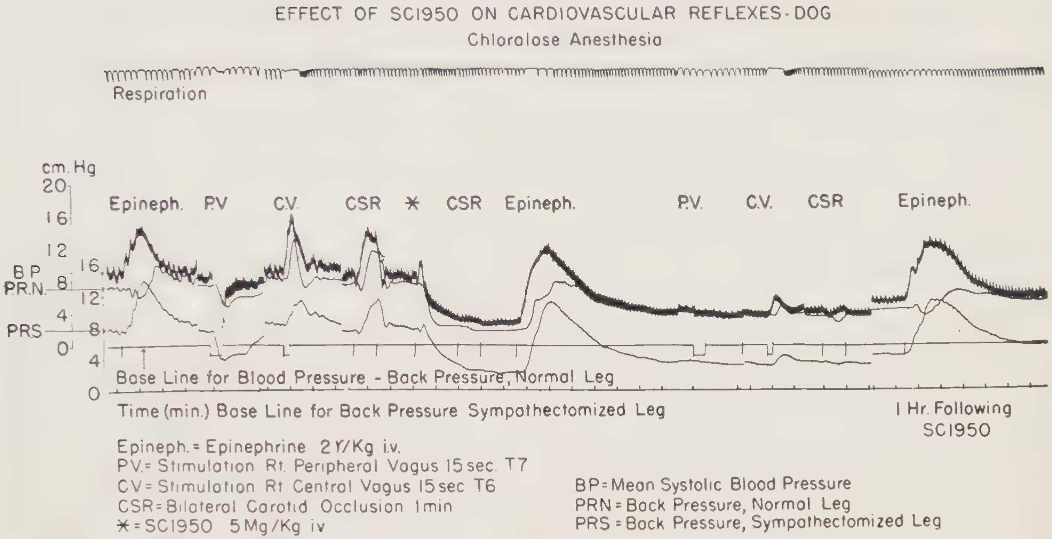

FIG. 2.

Systemic blood pressure and back pressures in normal and sympathectomized extremities (see text) are shown in response to epinephrine, peripheral and central vagal stimulation, and bilateral carotid occlusion, before and after 5.0 mg/kg SC 1950.

After 5 mg or more of SC 1950 the increase of blood pressure was blocked during all tests on 3 dogs. In the fourth dog this response was reduced but not abolished following 5 mg/kg and again following 10 mg/kg 2 hours later. Normally stimulation of the distal end of the divided right vagus effected cardiac slowing or arrest and fall of blood pressure. In 3 animals after SC 1950, 5 to 20 mg/kg, this response was completely blocked, heart rate continuing unchanged during vagal stimulation. In a fourth 5 mg/kg reduced but did not prevent cardiac slowing. Ten mg/kg 90 minutes later, however, prevented slowing. Anoxia produced an elevation of blood pressure of 20 mm Hg before the drug in only one of these 4 animals. After the drug this response did not occur. It is of interest that following removal of the gas an overshoot of blood pressure occurred once before and once after the drug. In the 3 dogs remaining small amounts of SC 1950 (0.02 to 1.0 mg/kg) did not block reflexes.

Blood pressure measured in the distal ends of the divided femoral arteries, representing "back pressure" or, indirectly, peripheral resistance, was lower in the sympathectomized extremity and responded slowly or passively in this limb during reflexes before the drug. Following SC 1950 peripheral or back pressure in the normal extremity decreased to equal that in the sympathectomized limb, and responses to cardiovascular reflexes were equally slow and passive.

(3) *Effect on response of blood pressure to intravenous epinephrine.* Epinephrine, 2 to 4 γ/kg, injected intravenously normally produced blood pressure increases ranging from 30 to 56 mm Hg following which the pressure returned to preinjection levels in two minutes or less. Following administration of SC 1950, 5 to 10 mg/kg, and during the associated reduction of systemic blood pressure, pressor responses to equal doses of epinephrine increased, reaching 46 to 168 mm Hg, a response 2 to 4 times as great as that before SC 1950 (Fig. 2). The duration of the pressor response also increased, ranging from 3.5 to 7.0 minutes following SC 1950 as compared with 2 minutes or less prior to the drug. Even after systemic blood pressure had returned to normal levels a half hour or more after injections of SC 1950, increased and prolonged responses to epinephrine occurred. Before SC 1950 back pressure of the normal leg decreased immediately following intravenous epinephrine and then increased. Initial decreases did not occur in the sympa-

thectomized leg. After the drug such initial decreases no longer occurred in the normal leg. The maximum increase of back pressure or "peripheral resistance" occurring after epinephrine was not as great in the normal extremity as in the sympathectomized extremity either before or after SC 1950.

(4) *Reversal of SC 1950 by neostigmine.* In 4 instances 0.5 to 1.0 mg neostigmine methylsulfate was administered intravenously after doses of 5 to 20 mg/kg SC 1950 had produced the above described reduction of blood pressure and block of reflexes. In each instance blood pressure rose promptly to levels near those before SC 1950, and the vascular reflexes again became active after neostigmine.

(5) *Effect on response to increased intracranial pressure.* Increase of intracranial pressure was effected by injection of saline into the skull through a trocar in 4 dogs anesthetized with chloralose. Intracranial pressure was kept greater than mean systolic blood pressure until death occurred. In normal animals[4] the terminal or agonal increase of blood pressure usually exceeds 200 mm Hg. This hypertensive response was not reduced in 3 dogs given 5 to 15 mg/kg of SC 1950, an amount adequate to block the pressor response to occlusion of the carotid arteries. The response to increased intracranial pressure was partially blocked in the fourth, receiving 15 mg/kg. The increases of blood pressure during increase of intracranial pressure after SC 1950 were respectively 58 to 218, 100 to 280, 54 to 204, and in the fourth dog 40 to 98 mm Hg.

(6) *Effect on pulse and blood pressure of unanesthetized dogs.* SC 1950, 2 to 15 mg/kg, was given intravenously to trained dogs whose blood pressure had been measured frequently by puncture of a femoral artery using a needle connected to a mercury manometer. Some of these dogs had been made hypertensive 3 months to one year previously by excision of the carotid sinuses and division of the cardio-aortic depressor nerves.[5] Others were normal animals without hypertension. Mean systolic blood pressure in the normal dogs ranged from 112 to 140 mm Hg (average 126) before the drug. In 12 tests on these normal dogs 2 to 15 mg/kg SC 1950 was injected intravenously. There were moderate increases of blood pressure in 11, blood pressures after the drug ranging between 130 and 162, the average being 147 mm Hg. Two of these dogs developed an initial rise of less than 20 mm Hg followed by a fall of pressure to below normal. One dog only had an immediate depressor response, blood pressure falling from 126 to 104 mm Hg. The moderate rise of blood pressure occurring after the drug lasted an average time of 36 minutes. All normal unanesthetized dogs developed increases of pulse rate following SC 1950, pulse rates before drug being 72 to 116 per minute, average 88, and after drug being 112 to 168, average 142 per minute.

In 11 tests on dogs made hypertensive by excision of the carotid sinuses and division of the depressor nerves, blood pressure before SC 1950 ranged from 198 to 264, average 223 mm Hg. Intravenous injection of 2 to 10 mg/kg SC 1950 consistently caused a decrease of blood pressure to levels ranging from 76 to 154, average 127. This reduction of blood pressure lasted 30 to 230 minutes, averaging 115 minutes. Pulse rates of these dogs ranged from 104 to 248, average 180 before the drug. Afterward the rate ranged from 84 to 144, average 114.

B. *Effect on the Gastrointestinal Tract.* Fluoroscopic and roentgenographic studies of the gastrointestinal tracts of 11 normal dogs were undertaken following administration of 30 g of barium sulfate in a 100 cc aqueous suspension by stomach tube. Fluoroscopy was performed immediately after administration of the barium observing gastric activity and timing the interval between administration of barium and initial emptying of the stomach. Roentgenograms were obtained 30 and 60 minutes following ingestion of barium and at hourly intervals thereafter until 6 hours had elapsed. Before the drug gastric peristalsis was active in all animals. Emptying of barium into the duodenum began in from less than one minute to slightly less than 8 minutes. Average time of initial emptying was 4.1 minutes in 11 animals. Time required for complete emptying of barium from the stomach varied markedly in a few normal animals but in most the emptying times were similar. Average amount of emptying at ½ hour was 30%, at 1 hour 46%, and at two hours 84%. Gastric emptying was complete at 3 hours in 8 of 10 dogs and at 4 hours in 9 of 10. Of 9 dogs in which observations could be made with accuracy, the first of the barium had traveled through the ileum and reached the colon at 2 hours in 3 dogs, and at 3 hours in 6 dogs.

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[4] Grimson, K. S., Wilson, H., and Phemister, D. B., *Ann. Surg.*, 1937, **106**, 801.

[5] Grimson, K. S., *Arch. Surg.*, 1941, **43**, 284.

On another day these control animals were given SC 1950 intravenously and barium sulfate by stomach tube as before 10 minutes following drug. Doses of 0.4 to 1.0 mg/kg were used in 5 animals. These small doses caused an acceleration of initial gastric emptying in 4, all beginning to empty in less than 2 minutes, and no change in the fifth. In the first 4 dogs subsequent emptying of the stomach was also accelerated, averaging 68% in ½ hour, 88% in 1 hour and 95% in 2 hours. The fifth dog had a normal emptying rate. The first barium reached the colon in 2 hours in 3, and 3 hours in 2.

Larger doses, 5 and 10 mg/kg, were then administered to 6 dogs. In each of these animals there was a definite delay of time of initial emptying and of total emptying. In 4 cases transit time through the small intestine was also prolonged. The time of initial emptying varied from 10 minutes to more than an hour, average 29 minutes. At ½ hour 4 animals showed no emptying, one 1% emptying, and one 20% emptying. By 1 hour average emptying was 17%, one animal still having complete gastric retention and 2 others having passed only traces of barium into the small intestine. At 2 hours emptying averaged 60%, at 3 hours 71%, and at 4 hours 82%. In the 4 dogs receiving 10 mg/kg SC 1950, barium traversed the ileum and reached the colon only after 4 to 6 hours. In the 2 receiving 5 mg/kg, barium appeared in the colon at 2 and 3 hours.

Fluoroscopy following the smaller doses (0.4 to 1.0 mg/kg) of SC 1950 revealed little change in gastric motility from the control animals. Following larger doses peristalsis was markedly decreased in 5, but appeared normal in one although initial and total emptying was delayed.

II. *Action of SC 1950 in Man.* Doses of SC 1950 varying from 0.5 to 2.0 mg/kg or 31 to 150 mg per patient were administered intravenously and of 2.0 mg/kg or 100 to 192 mg per patient intramuscularly to 53 patients without evident ill effects and without subsequent abnormality evident in blood counts or urinalysis. Immediately after intravenous injection patients had reddening of the skin and experienced a feeling of warmth. Most had a sense of general relaxation and some became slightly drowsy. Within a few minutes dryness of mucous membranes, dilatation of pupils, and loss of accommodation were noted. Occasionally ptosis of the upper eyelids and engorgement of conjunctival blood vessels appeared. Most of these changes persisted 30 to 60 minutes after intravenous injection. Following intramuscular administration these effects of the drug were noted in approximately 15 minutes and persisted 2 to 3 hours. One patient had pain from a duodenal ulcer at the time the drug was given and experienced relief for 1½ hours following 1.0 mg/kg intravenously. Another had right upper quadrant pain persisting 3 months following cholecystectomy and experienced relief for 110 minutes following 1.5 mg/kg. A burning pain of the foot in another, evidently associated with diabetic neuropathy, was repeatedly relieved for several hours following injections of 1.5 mg/kg. Several patients experiencing nausea before SC 1950 were relieved immediately after its injection. Oral administration of 2 to 15 mg/kg (100 to 900 mg total dose) produced only slight and inconsistent effects.

A. *Effect on Cardiovascular System of Man.* With the exception of one patient who had slight pressor responses with repeated doses of SC 1950, 0.5 to 1.5 mg/kg, lowering of blood pressure was consistently produced by the drug. The decrease following intravenous injection in normotensive patients in the supine position was from an average of 125/77 before the drug to an average of 94/62 afterward. In hypertensive patients in the supine position the reduction was from an average of 190/130 before the drug to 114/80 afterward. The greatest fall of blood pressure occurred within two minutes following intravenous injection, following which pressures gradually rose to their former levels in from 10 to 120 minutes, usually about ½ hour. With standing, decrease of blood pressure (postural hypotension) was more pronounced. This postural hypotension occurred immediately after the drug and could be demonstrated a half hour or longer following return of supine blood pressure readings to levels equal to those before the drug.

Pressor responses normally occurring with immersion of the hand in ice water for 60 seconds and with breath holding were tested before and after SC 1950 in 12 patients. In each of the 12 pressor responses were obtained with both stimuli before the drug. After 1.0 to 1.5 mg/kg pressor responses to cold were prevented or reversed in 9 cases, reduced in 2, and still active in one. Pressor responses to breath holding were blocked or reversed in 8 patients, and reduced in 4.

The temperature gradients of lower extremities were tested at a constant room temperature of 25° C before and after 1.0 to 2.0 mg/kg SC 1950 in 12 patients. Skin temperatures were obtained with a Cambridge thermocouple. Before the drug toes were several degrees colder than the umbilicus. Afterward in 9 patients the gradient was completely abolished, warmth of toes and feet equaling that at the umbilicus. In one patient with arteriosclerotic occlusive vascular disease gradient was reduced but not abolished. Another with marked arteriosclerotic arterial insufficiency had no change of gradient. The final patient had no change of gradient. She weighed only 44 kg and had only 66 mg total dose of SC 1950.

As a rule cadiac rate was not markedly affected by SC 1950. Most patients had a slight tachycardia during the hypotensive action of the drug. A few patients with tachycardia before SC 1950 had moderate cardiac slowing following the drug.

B. *Effect on Human Gastrointestinal Activity.* Balloon studies of activity of the stomach and small intestine were performed in 20 patients. Gastric motility was studied using a full-sized condom fastened over the tip of a No. 14F Levine tube. Balloon tension or pressure at 300 cc was first recorded on the kymograph. The balloon was then deflated and passed with the tube into the stomach, placing the tip approximately 5 inches past the cardia under fluoroscopic guidance. The Levine tube was connected to a bromoform manometer and the balloon then reinflated to 300 cc by 50 cc increments of air. Rate of respirations was recorded by a pneumograph attached to the chest of the patient and connected to a tambour. In 2 patients studies of activity of the small intestine were made with a small balloon passed into the intestine on a Miller Abbot tube and inflated to 20 cc. In 3 more detailed studies were carried out employing the 4 lumen tube method described by Chapman[6] with the proximal balloon in the stomach or upper duodenum and 3 other balloons 8 inches apart distributed in the duodenum and jejunum.

Intravenous administration of 1.0 to 1.5 mg/kg SC 1950 consistently caused an immediate decrease of resting intragastric pressure or "tone" and cessation of forceful fluctuation of pressure or "peristalsis" of the stomach and the small intestine. The "tone" of the stomach began returning 25 to 40 minutes following SC 1950 and after 60 to 80 minutes tone was often slightly greater than before the drug. Gastric peristaltic activity was entirely absent for 30 minutes or more in all cases, and an average time of 58 minutes elapsed before any return of contractility was noted. Complete recovery of gastric activity was never regained in less than one hour, and usually 1½ hours were required before contractions became relatively normal.

Observations of activity of the small intestine closely paralleled those described for the stomach, reduction of tone and cessation of contractions occurring after the drug. When the 4 lumen tube was used it was noted that increased tone and contractions reap-

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[6] Chapman, W. P., Stanbury, J. B., and Jones, C. M., *J. Clin. Invest.*, 1948, **27**, 34.

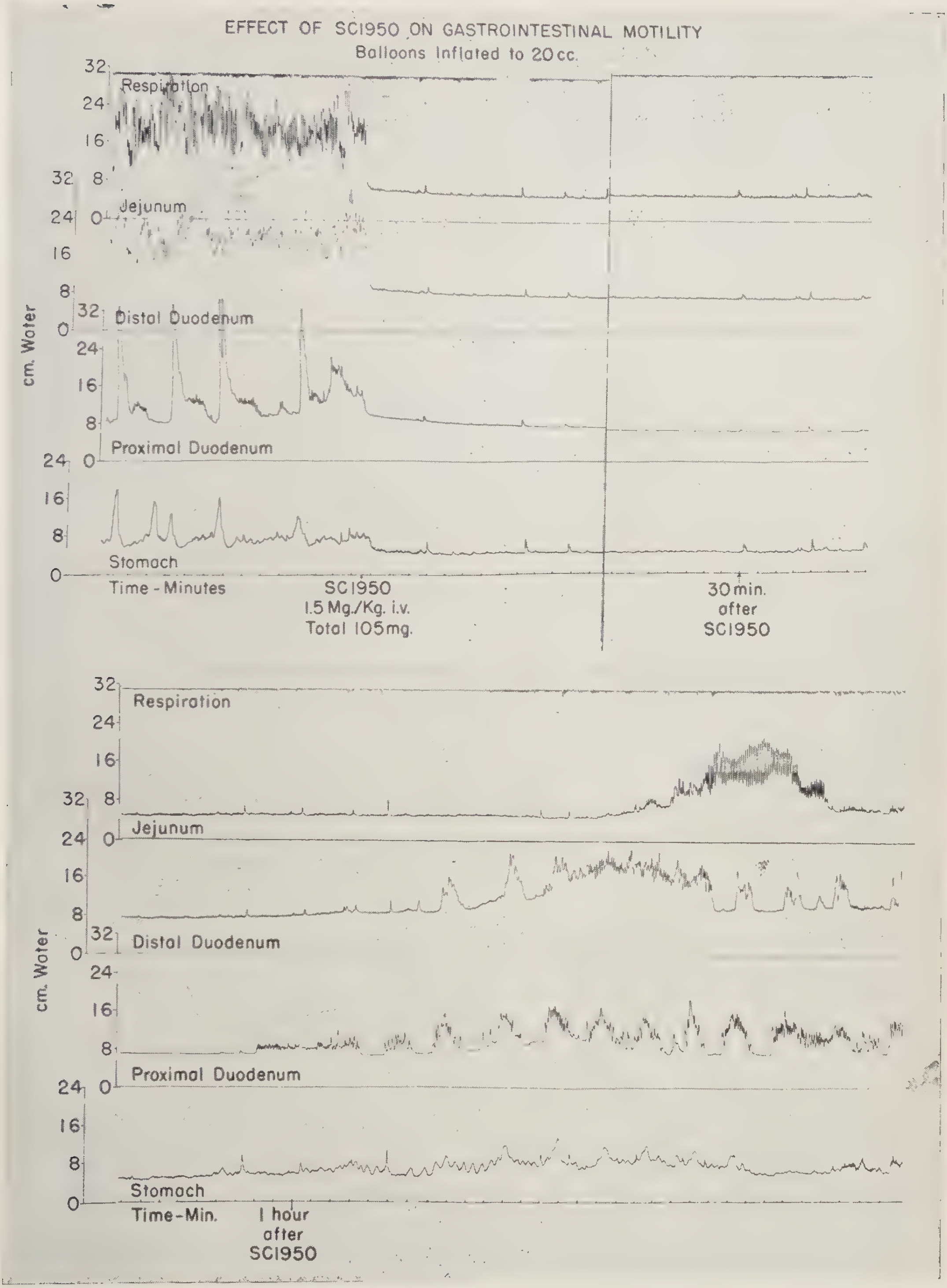

Fig. 3.

Activity of the stomach and upper small intestine as measured by 4 balloons 8 inches apart. SC1950 reduces tone and stops contractions for a period of one hour. Recovery occurs first in the more proximal segments.

TABLE I.
Effect of Urecholine and Prostigmine on Human Gastric Activity Following SC 1950 I.V. Dose 1.5 mg/kg. Gastrometric Studies. (300 cc Balloon.)

Gastric activity following SC 1950	Interval before second drug (min.)	Second drug	Total dose, No. mg	Route	Recovery time (min.)	
					Following second drug	Following SC 1950
0	12	Urecholine	2.5	I.M	20	32
0	12	”	2.5	”	19	31
0	13	”	4.0	”	15	28
0	5	”	5.0	”	6	11
0	7	Prostigmine	1.0	”	41	48
0	11	”	0.5	”	23	34
0	12	”	0.5	I.V.	8	20
0	10	”	0.5	”	11	21

peared first in the balloon located most proximally and then successively in the more distal balloons. Usually 6 to 8 minutes elapsed before activity appeared in successive segments 8 inches apart (Fig. 3). Four to 10 minutes after resumption of peristaltic activity the small intestine occasionally became excessively active for a period of 8 to 10 minutes. The duration of the period of reduced tone and absence of contractions following doses of 1.5 mg/kg SC 1950 was with one exception an hour or more and averaged 72 minutes. The exception occurred in a patient weighing only 40 kg, receiving a total dose of 80 mg. Total cessation of activity in this patient lasted 40 minutes. In the small intestine normal contractions appeared within a few minutes of resumption of activity.

Four subjects were given 1.5 mg/kg SC 1950 intravenously and 12 minutes later were given Urecholine 2.5 to 5 mg intramuscularly. In each patient SC 1950 produced lowering of tone and complete cessation of activity as measured by gastric balloons (Table I). After Urecholine all developed a slight increase of tone within 10 minutes and all had resumption of peristaltic activity within 20 minutes following Urecholine, or 11, 28, 31, and 32 minutes after SC 1950. As described above the average time of return of peristalsis following SC 1950 alone was 58 minutes, only one patient of 8 showing return of gastric activity 30 minutes following administration. Four other gastric motility studies were done giving neostigmine 0.5 mg 7 to 12 minutes after tone had been decreased and contractions abolished by 1.5 mg/kg of SC 1950 given intravenously. In 2 of these studies 0.5 mg neostigmine was given intravenously and in 2 intramuscularly. Motility returned 8 and 11 minutes following intravenous neostigmine or 20 and 21 minutes following SC 1950 (Table I). In one of 2 patients receiving 0.5 mg neostigmine intramuscularly, motility returned 23 minutes following neostigmine and 34 minutes following SC 1950. The other subject received two intramuscular injections of 0.5 mg neostigmine, the first 7, the last 22 minutes following 1.5 mg/kg SC 1950 intravenously. No recurrence of contractions was noted until 41 minutes following the first neostigmine or 48 minutes following the SC 1950.

Six patients with apparently normal gastrointestinal tracts had fluoroscopic and roentgenologic studies performed after ingestion of barium. Five of these had control studies in which 120 g barium sulfate suspended in 200 cc water was given orally. Fluoroscopy was done immediately and gastric activity and rate of emptying were noted. Roentgenograms were obtained at hourly intervals for 6 hours following ingestion. No abnormalities were noted. Gastric peristalsis was evident in all immediately after ingestion of barium and emptying into the duodenum occurred promptly. At one hour gastric emptying was at least 50% complete in all, average being 70% (Fig. 4A). The most distal barium was in the ileum at one hour in all. Two hours after ingestion 98%-100% of barium had passed through the stomach, and the most distal barium had advanced to the midileum, terminal ileum,

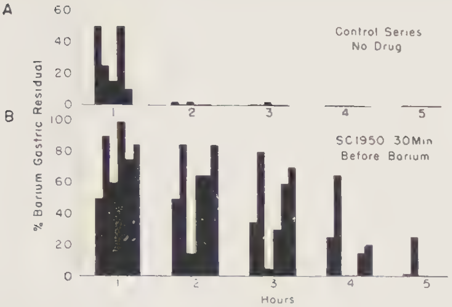

FIG. 4.

Roentgenographic study of the human gastrointestinal tract. Gastric emptying before and after 2 mg/kg SC 1950 IM.

or cecum. Barium had reached the cecum at 1, 2, 3, 3, and 4 hours respectively.

On another day SC 1950 was given to these same patients in amounts of 2 mg/kg intramuscularly, 30 minutes before ingestion of barium. Following SC 1950 and immediately after giving barium no peristaltic activity was noted by fluoroscopy in any patient. Only one demonstrated slight emptying immediately after barium. At one hour only one patient had as much as 50% emptying, and average emptying was 25%. At 2 hours there was a 40% average emptying with variations from 15% to 85% (Fig. 4B) Also at 2 hours the most distal barium was in the duodenum or jejunum in 5 patients and in the upper ileum in one. At 4 hours 2 of 6 had 100% gastric emptying and at 6 hours 4 of 6 had 100% emptying. By 4 hours barium had reached the cecum in all control patients but in only one of 6 patients after SC 1950.

C. *Effect on Gastric Secretions.* Preliminary studies have been performed on 4 patients testing effect of SC 1950 on volume and acidity of gastric secretions. Continuous gastric aspiration was employed, and samples were taken every 15 minutes. These patients all had duodenal ulcers and secreted large volumes of highly acid gastric juice. Following SC 1950 (1.5 mg/kg) intravenously, volume and acidity of gastric secretions was markedly diminished for an hour or more. Free acid was present in all specimens prior to SC 1950. Afterward there was no free acid in 2 or more specimens from 3 patients. In the fourth the total acid secreted was reduced from an average of 0.51 m eq during each 15 minute period to an average of 0.09 m eq during each 15 minute period. Although free acid never disappeared, this reduction of acidity following SC 1950 persisted 1¼ hours.

Conclusion. A new quaternary amine, 2,6, dimethyl, diethyl piperidinium bromide, described as a ganglionic blocking agent,[4] has an inhibitory or blocking action upon functions mediated by the sympathetic and parasympathetic divisions of the autonomic nervous system. In dog and in man it reduces blood pressure and prevents several vasomotor reflexes, but does not prevent the vasopressor action of epinephrine. It causes a reduction of tone of the stomach and small intestine and abolishes their motor activity, reduces gastric acidity, and causes pupillary dilatation, loss of accommodation, and dryness of mucous membranes. Urecholine causes a reversal of effect on gastric motility. Neostigmine apparently causes a reversal of all effects of the drug.

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Specificity of Differential Sheep Cell Agglutination Test in Rheumatoid Arthritis.*

S. EDWARD SULKIN, ROBERT M. PIKE, AND HOWARD C. COGGESHALL.

From the Department of Bacteriology and Immunology, and the Department of Medicine, Southwestern Medical College, Dallas.

Rose and his associates[1] recently described a sheep cell agglutination test which may prove to be helpful as an aid in the differential diagnosis between rheumatoid arthritis and other diseases with arthritic manifestations. These authors found that a 16-fold difference or greater between agglutination titers with sensitized and unsensitized sheep erythrocytes occurred almost exclusively with sera from patients with rheumatoid arthritis. The differential agglutination titer usually reflected the clinical activity of the disease. Although the serologic properties of the sera of rheumatoid arthritis patients have been extensively studied, no test of definite diagnostic value has been devised.[2-5] It therefore seemed desirable to evaluate the differential sheep cell agglutination test.

Methods and Materials. In performing the test, the procedure described by Rose and his associates[1] was followed in detail. Tests were read immediately upon removal from the refrigerator. The end point of each titration was read as the highest serum dilution showing one-plus agglutination. The titer was recorded as the reciprocal of that serum dilution. The differential agglutination titer as presented in the accompanying tables represents the difference in titers obtained in tests conducted with unsensitized and with sensitized sheep erythrocytes and is recorded as the algebraic difference between these titers.

The test was performed with serum from 43 patients with rheumatoid arthritis including 7 patients with rheumatoid spondylitis (Marie-Strümpell) and one patient with juvenile rheumatoid arthritis (Still's disease). In all but one of these patients the disease was considered clinically active. For purposes of comparison, sera from 88 individuals with a variety of other conditions including diseases with and without evidence of arthritis were examined. Also included were serum specimens from 18 presumably normal persons.

Results. The results of the differential sheep cell agglutination test performed with serum specimens from 148 individuals are summarized in Table I. It is evident from these data that serum specimens from only 17 of the 42 patients with rheumatoid arthritis showed differential agglutination titers of 16 or greater. These were all from clinically active cases. Thus, these observations confirm, in part, those of Rose and his associates.[1] There were 24 cases of active rheumatoid arthritis and one inactive case of long duration in which a differential agglutination titer of less than 16 was obtained. In this respect these observations differ from those previously recorded.[1] Of the remaining 106 serum specimens from individuals with a variety of conditions, including 18 presumably normal persons, none showed a differential sheep cell agglutination titer greater than 8 and all except 5 had titers less than 8. The serum from only one of 18 patients with acute rheumatic fever (juvenile) showed a differential titer as great as 4. Fourteen cases of lymphogranuloma venereum which had been proved by the Frei skin test and by complement-fixation with a yolk sac antigen were included because of the reported occurrence of increased titers of sheep cell agglutinins in this disease.[6†] None of the serum specimens from these patients showed a differential titer greater than 4.

Table II summarizes the results of the differential sheep cell agglutination test in the group of patients with rheumatoid arthritis exclusive of those with spondylitis. All but one of the patients showed evidence of active joint disease at the time specimens were obtained. In the tabulation, the cases are classified, without knowledge of the results of the agglutination test, according to the severity and duration of the illness. The following were taken into consideration when classify-

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[*] Aided in part by grants from the Lederle Laboratories Division of the American Cyanamid Company, Pearl River, N. Y., and the Rose Lampert Graff Foundation, Los Angeles, Calif.

[1] Rose, H. M., Ragan, C., Pearce, E., and Lipman, M. O., Proc. Soc. Exp. Biol. and Med., 1948, **68**, 1.

[2] Nicholls, E. E., and Stainsby, W. J., *J. Clin. Invest.*, 1931, **10**, 323.

[3] Dawson, M. H., Olmstead, M., and Boots, R. H., *J. Immunol.*, 1932, **23**, 187.

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[6] Sales Gomes, L., and Brito e Silva, M., *Revista do Inst. Adolfo Lutz,* 1942, **2**, 212; *ibid.*, 1943, **3**, 25.

[†] This observation, incidentally, has not been confirmed in this laboratory.

TABLE I.

Results of Differential Sheep Cell Agglutination Tests on Serum Specimens from Patients with Rheumatoid Arthritis and a Variety of Other Conditions.

Clinical diagnosis*	No. of cases	Distribution of cases according to differential agglutination titer									
		0	2	4	8	16	32	64	128	256	512
Rheumatoid arthritis	35	1	8	6	4	6	5	4			1
Rheumatoid spondylitis (Marie-Strümpell)	7		4	1	1†	1†					
Reiter's disease	1		1								
Arthralgia	1		1								
Osteo-arthritis	3	1	1		1						
Gonorrheal arthritis (?)	1		1								
Scleroderma	3		1		2						
Lupus erythematosus disseminatus	1		1								
Acute rheumatic fever	18	9	8	1							
Infectious mononucleosis	2	2									
Lymphogranuloma venereum	14		11	3							
Syphilis	17	5	10	2							
Infectious diseases without evidence of arthritis‡	18	4	9	5							
Other diseases without evidence of arthritis§	9	2	6	1							
Presumably normal persons	18	3	13	10	2						

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* In all instances the clinical diagnosis was substantiated by appropriate laboratory tests when availble.

† This patient also had peripheral joint involvement.

‡ These include one case each of brucellosis, infectious hepatitis, equine encephalomyelitis (Eastern type), primary atypical pneumonia, typhus fever, pneumococcal pneumonia, and poliomyelitis, 2 cases each of psittacosis and toxoplamosis, 3 cases of lymphocytic choriomeningitis, and 4 cases of Q fever.

§ These include one case each of acute glomerulonephritis and Hodgkin's disease, 2 cases each of myelogenous leukemia, carcinoma and multiple myeloma.

TABLE II.

Results of Differential Sheep Cell Agglutination Tests on Serum Specimens from Patients with Rheumatoid Arthritis Classified According to Severity and Duration of Illness.*

Severity of disease	Duration of illness (yrs)	No. of cases	Distribution of cases according to differential agglutination titer									
			0	2	4	8	16	32	64	128	256	512
Mild	<1	2			2							
	1–5	3		2	1							
	>5	0										
Moderate	<1	3	1	1	1							
	1–5	10		2		4	2	2				
	>5	8		1	2		4		1			
Marked	<1	0										
	1–5	2						1	1			
	>5	7		2†				2	2			1

* Not including 7 cases with evidence of rheumatoid spondylitis (Marie-Strümpell).

† One of these is an inactive case of long duration, the other is a case of juvenile rheumatoid arthritis (Still's disease).

ing these patients: intensity of constitutional symptoms, speed of progression of illness, degree of disability, degree and extent of joint swelling, amount of bone atrophy and destruction, and activity evidenced by results of erythrocyte sedimentation tests.

It is evident from the data presented in Table II that none of the patients classified as having mild rheumatic arthritis and none of those with a disease of moderate severity of less than one year duration showed differential agglutination titers greater than 4. Only 2 patients with a disease of marked severity had a differential agglutination titer less than 32. One of these patients with rheumatoid arthritis for a period of 30 years was "inactive" when the blood specimen was obtained. The other was a case of juvenile rheumatoid arthritis (Still's disease). Thirteen of the 18 cases with an illness of moderate severity of more than one year duration had differential agglutination titers of 8 or greater. The distribution of cases according to the differential agglutination titer would seem to reflect the clinical severity of the disease.

Many of the patients with high differential agglutination titers also had high sedimentation rates. There was no correlation, however, between the height of the sedimentation rate and the result of the differential sheep cell agglutination test, since some patients whose serum showed a differential agglutination titer of less than 16 had high sedimentation rates. This, likewise, is in accord with observations reported previously.[1]

A second blood specimen was obtained from 5 of the patients with rheumatoid arthritis after an interval of about one month. One original differential agglutination titer of 512, one of 64, and two of 4 were found to be the same, while one titer of 16 was 8 on the second examination. Further follow-up studies are contemplated with the view to elucidating the mechanism of this phenomenon. Data thus far available indicate that the differential sheep cell agglutination test is of limited value as an aid in the diagnosis of active rheumatoid arthritis of mild severity.

Summary. 1. The differential sheep cell agglutination test of Rose and his associates was performed with serum specimens from 42 individuals with rheumatoid arthritis. Serum from 17 of these patients showed differential agglutination titers of 16 or greater. In 24 active cases of mild and moderate severity and in one inactive case of long duration a differential agglutination titer of less than 16 was obtained.

2. Sera from 88 individuals with a variety of other conditions and from 18 presumably normal persons tested in the same manner showed differential agglutination titers less than 16.

3. Although differential agglutination titers less than 16 were observed in more than half the cases of active rheumatoid arthritis, the

test appears to reflect the clinical severity of the disease.

4. These data partially confirm the observations of Rose and his associates but indicate that the differential sheep cell agglutination test is of limited value as an aid in the diagnosis of active rheumatoid arthritis of mild severity.

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IV. Ineffectiveness of Certain Essential Nutrients in Prevention of Tooth Decay in Cotton Rat Molars.

James H. Shaw.

From the School of Dental Medicine and Department of Nutrition, School of Public Health, Harvard University, Boston, Mass.

The molar teeth of cotton rats (*Sigmondon hispidus hispidus*) have been found to be susceptible to the initiation and development of carious lesions when the rats were maintained on purified rations for 10 weeks or more after weaning.[1,2] The basal rations used in these studies were believed to be adequate in known essential nutrients in so far as the growth and maintenance of rodents were concerned. When the fat, or the protein content, or both, were increased isocalorically at the expense of sucrose, weanling cotton rats fed the resulting rations developed a much lower average number and average extent of carious lesions than the littermates fed the basal ration for the same period.[3,4] Before extensive use of this ration and its various modifications in the investigation of the effect of diet during or after tooth development on the susceptibility to tooth decay, it seemed mandatory to determine whether superabundant amounts of known essential nutrients, or the same nutrients from different sources, would alter the initiation and development of carious lesions in cotton rats when the molar teeth were fully formed and were known to be highly susceptible to dental caries at the beginning of the experimental period.

Experimental. The purified ration (100) used in most of these studies has been described in detail.[5] In a few of these studies, a slight modification (130) of the basal ration was used which was made by the isocaloric replacement of 18 of each 67 g of sucrose with 8 g of lard. Both diets were nutritionally adequate for the production of normal growth and development of the cotton rat.

The cotton rats used in these investigations were raised in our stock colony and weaned at about 21 days of age. At this time, the first and second molars of the cotton rat are fully erupted and the crowns of the third molars are largely formed but have not erupted into the oral cavity. Experimental procedure was identical with that described for previous experiments.[5]

In the first experiment, a series of 7 groups of cotton rats was fed various combinations of water-soluble and fat-soluble vitamins in superabundant amounts as described in Table I. Parallel to each experimental group, a comparable group of control littermates was maintained on ration 100, except in the seventh group where ration 100 without liver concentrates was used.

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[2] Shaw, J. H., Schweigert, B. S., Elvehjem, C. A., and Phillips, P. H., *J. Dental Research*, 1944, **23**, 47.

[3] Schweigert, B. S., Shaw, J. H., Zepplin, M., and Elvehjem, C. A., *J. Nutrition*, 1946, **31**, 439.

[4] Schweigert, B. S., Potts, E., Shaw, J. H., Zepplin, M., and Phillips, P. H., *J. Nutrition*, 1946, **32**, 405.

[5] Shaw, J. H., *J. Dental Research*, 1947, **26**, 47.

TABLE I. Effect of Additions of Various Water-Soluble, Fat-Soluble Vitamins on Average Number, Average Extent Carious Lesions in Cotton Rat.

Ration No.	No. of cotton rats	Avg No. of carious lesions	S.E.M.*	C.R.†	Avg extent of carious lesions	S.E.M.*	C.R.†
100	7	29.6	2.1	.3	79+	10+	.4
100 + B-complex‡	7	28.9	1.8		86+	12+	
100	5	26.8	1.7	1.2	83+	7+	1.8
100 + 0.4% ascorbic acid	5	23.6	2.1		65+	7+	
100	5	24.8	4.2	.6	67+	18+	.2
100 + B-complex‡ + 0.4% ascorbic acid	7	27.6	2.7		71+	16+	
100	10	26.1	2.4	.3	73+	11+	.4
100 + ADEK§	11	27.1	2.6		78+	10+	
100	4	24.5	2.6	.9	74+	12+	.6
100 + 0.1% menadione	5	27.2	1.4		84+	9+	
100	9	33.0	1.0	1.2	110+	6+	.6
100 + 0.1% natural vitamin K_1	10	31.3	1.0		104+	8+	
100	12	26.7	1.1	.3	75+	6+	.4
100 + liver concentrates + 200 μg folic acid	11	27.2	1.0		79+	7+	

* Standard error of mean. † Critical ratio.

‡ To each kg of ration 100 were added an additional 10.5 mg of thiamine hydrochloride, 10.5 mg of riboflavin, 10.5 mg of pyridoxine hydrochloride, 75 mg of nicotinic acid, 60 mg of calcium pantothenate, 3.0 g of choline chloride, 3.0 g of inositol, and .9 g of para-aminobenzoic acid, *i.e.*, enough of each to make the final concentration 4 times the amounts normally supplied. In addition, 200 μg of biotin were added per kilo.

§ To each kg of ration 100 were added an additional 33 mg of beta-carotene, 9000 I.U. of irradiated ergosterol, 18 mg of 2-methyl, 1,4-naphthoquinone, and 150 mg of alpha-tocopherol, *i.e.*, enough of each to make the final concentration 4 times the amounts ordinarily supplied.

TABLE II. Effect of the Replacement of Purified Casein by Crude Casein, Blood Fibrin, and Lactalbumin and of Additions of *dl*-Tryptophane on the Average Number and Average Extent of Carious Lesions in the Cotton Rat.

Ration No.	No. of cotton rats	Avg No. of carious lesions	S.E.M.*	C.R.†	Avg extent of carious lesions	S.E.M.*	C.R.†
100	4	37.0	.9	.3	120+	6+	1.2
400 (crude casein)	4	35.8	3.0		104+	10+	
130	3	24.3	3.8	.7	57+	17+	.3
430 (crude casein)	3	21.0	2.9		51+	13+	
100	8	29.5	1.3	2.0	83+	7+	2.0
200 (blood fibrin)	8	32.5	.7		102+	6+	
130	8	18.0	2.5	.5	41+	10+	.3
230 (blood fibrin)	9	17.3	2.3		36+	8+	
100	10	32.2	1.2	.9	104+	7+	1.1
300 (lactalbumin)	10	30.1	1.9		90+	10+	
130	6	19.5	1.9	1.5	41+	9+	1.1
330 (lactalbumin)	6	24.5	2.7		57+	11+	
100	9	27.8	2.0	1.1	77+	9+	.6
100 + 0.5% *dl*-tryptophane	9	25.2	1.4		70+	8+	

* Standard error of mean. † Critical ratio.

TABLE III.
Effect of Mineral Alterations on the Average Number and Average Extent of Carious Lesions in the Cotton Rat.

Ration No.	No. of cotton rats	Avg No. of carious lesions	S.E.M.*	C.R.†	Avg extent of carious lesions	S.E.M.*	C.R.†
100	7	27.6	1.8	.9	85+	10+	.9
100 + 2% salt mixt.	7	25.7	1.1		75+	6+	
100	9	29.4	1.4	.6	96+	6+	1.1
1100 (sol. salt mixt.)‡	9	28.3	1.1		87+	6+	
100	6	28.7	1.9	1.2	86+	10+	1.2
1100 + 2% sol.‡ salt mixt.	5	24.4	3.1		69+	10+	
100	7	24.3	2.8	1.5	69+	11+	1.0
100 + 0.5% sodium oxalate	9	19.3	1.9		55+	8+	
100	5	23.8	1.9	1.9	64+	11+	1.0
100 + 1.0% sodium oxalate	6	18.2	2.2		48+	12+	

* Standard error of mean

† Critical ratio.

‡ Calcium lactate 490 g, anhydrous calcium chloride 710 g, monosodium phosphate 1050 g, anhydrous sodium carbonate 207 g, anhydrous potassium carbonate 480 g, anhydrous magnesium sulfate 100 g, ferric citrate 55 g, potassium iodide 1.6 g, manganese sulfate 9.0 g, anhydrous zinc chloride 4.0 g, anhydrous cupric sulfate 2.4 g, and cobaltous sulfate 0.2 g.

TABLE IV.
Average Number and Extent of Carious Lesions Observed in Cotton Rats Maintained for 14 Weeks on Ration 100, Keyes' Hamster Ration, and on Ration 100 Plus Yeast.

Ration No.	No. of cotton rats	Avg No. of carious lesions	S.E.M.*	C.R.†	Avg extent of carious lesions	S.E.M.*	C.R.†
100	4	33.5	1.5	5.3	106+	7+	7.0
Keyes'	5	16.8	2.6		36+	8+	
100-liver	7	33.7	1.4	3.0	110+	6+	2.1
100-liver + 8% brewer's yeast	8	28.0	1.5		89+	8+	

*Standard error of mean.

† Critical ratio.

In the second experiment, crude casein, blood fibrin, and lactalbumin were used as individual protein sources by the complete replacement of the "de-vitaminized" casein in ration 100, or in ration 130. In addition, the effect of a supplement of 0.5% *dl*-tryptophane to ration 100 was tested.

In the third experiment, a series of mineral alterations was made, as detailed in Table III.

In the fourth experiment, one group of experimental cotton rats was fed the ration* used by Keyes[6] for the production of carious lesions in the hamster. A second group of cotton rats was fed ration 100 with the liver concentrates replaced by 8% dried brewer's yeast.

Results. The results of the first experiment in which additional amounts of the water-soluble and fat-soluble vitamins were added to ration 100 throughout the 14-week experimental period are summarized in Table I. In all cases, the additional amounts of these nutrients tested, after development of the teeth was largely completed, did not significantly alter the average number nor the average extent of carious lesions. The rate of increase in body weight was not altered by any of these supplements.

The results of the second experiment, in which the "de-vitaminized" casein in rations 100 or 130 was completely replaced by crude casein, blood fibrin, or lactalbumin, and where ration 100 was supplemented by 0.5% *dl*-tryptophane, are presented in Table II. The use of crude casein, blood fibrin, or lactalbumin did not alter the susceptibility of these cotton rats to develop carious lesions under the circumstances in this experimental regimen. Likewise, a supplement of 0.5% *dl*-tryptophane did not alter the initiation and development of carious lesions when fed after tooth development was complete. Increases in body weight were not altered by these supplements, except in the case of those cotton rats fed blood fibrin. These animals gained slightly more rapidly during the early weeks of the experiment and attained slightly higher average final body weights.

The results of the mineral alterations in the third experiment are presented in Table III. A supplement of an additional 2% of the regular salt mixture, and the replacement of the regular salt mixture by a more soluble salt mixture at 3 and 5% did not alter the susceptibility to tooth decay when fed after tooth development was largely finished. The supplements of both 0.5 and 1.0% sodium oxalate to ration 100 seemed to reduce the average number of carious lesions and the average extent slightly, but not sufficiently to be judged statistically significant by the test used. Both groups of cotton rats fed sodium oxalate had a severe and prolonged diarrhea and did not gain weight as rapidly as their controls nor attain as high final body weights. These factors may have been responsible to some degree for any tendency to reduce the development of carious lesions.

When cotton rats were fed a ration described by Keyes[6] for production of a high incidence of carious lesions in the hamster, the average number and average extent of carious lesions were significantly lower than for littermates fed ration 100 during the same period (Table IV). Those lesions which did develop in cotton rats fed Keyes' ration were characteristic in location and in type with those observed in cotton rats after only 6 to 10 weeks on experiment. Unlike the hamster, no lesions were produced on the smooth surfaces of the molar teeth in the cotton rat. One possible explanation for the reduced dental caries attack rate in cotton rats fed Keyes' ration may be based upon the low food intake and low rate of growth observed. Due to the apparent dislike of this ration by cotton rats, more ration was scattered and wasted than was eaten. A similar effect of enforced food restriction on the initiation and development of carious lesions has been described previously.[7]

The replacement of the liver concentrates in ration 100 by 8% of brewer's yeast resulted in a small reduction, bordering on statistical significance in the average number and average extent of the carious lesions.

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* Whole wheat flour 20%, cornstarch 25%, confectionery sugar 20%, whole powdered milk 30%, and ground alfalfa 5%.

[6] Keyes, P. H., *J. Dental Research*, 1946, **25**, 341.

[7] Shaw, J. H., *J. Am. Diet. Assn.*, 1948, **24**, 181.

Summary. A variety of tests were made to determine if superabundant amounts of essential nutrients or optimal amounts of these nutrients from different sources would alter the initiation and development of carious lesions in caries-susceptible cotton rats after tooth development was almost complete. Negative results were obtained in all cases indicating that initiation and development of carious lesions in teeth formed before the beginning of the experimental period were not influenced by additional amounts of the known nutrients tested.

The ration described by Keyes for production of carious lesions in hamsters resulted in very poor growth and a low rate of initiation and development of carious lesions in the cotton rat. A supplement of 8% dried brewer's yeast to the purified ration produced good growth and permitted a slight reduction in the dental caries experience of cotton rats.

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Tuberculostatic Effect of Furacin *in vitro* and *in vivo*.*

E. WOLINSKY, VERNA WETZEL, AND W. STEENKEN, JR.
(Introduced by David T. Smith.)
From The Trudeau Laboratory, Trudeau Foundation for Clinical and Experimental Study of Pulmonary Disease, Trudeau, N. Y.

Furacin is a nitrofuran compound (5-nitro-2-furaldehyde semicarbazone) which has definite chemotherapeutic effects against certain infections *in vivo*, as well as bacteriostatic and bactericidal properties against gram-positive and gram-negative organisms in the test tube.[1-7]

This report presents the results of experiments designed to show the effect of Furacin on the growth of tubercle bacilli in the test tube, and on a tuberculous infection in guinea pigs.

To determine the influence of Furacin on the growth of the human type H37 Rv microörganisms, 4 different fluid media were employed: Proskauer and Beck's synthetic medium, Proskauer and Beck's medium with 10% human serum, Kirschner's synthetic medium, and the Tween-albumin medium of Dubos. They were dispensed in 5 cc amounts in 18x150 mm test tubes. The inoculum into each tube was 0.1 cc of a 7-day culture of H37 Rv microörganisms (about 0.03 mg dry weight) in Tween-albumin liquid medium. All determinations were made in triplicate.

Table I reveals that Furacin in a concentration of 1:40,000 produced a slight inhibition of growth of the organisms, whereas in a concentration of 1:20,000 it prevented any appreciable growth for 28 days.

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* The Furacin was supplied through the courtesy of Dr. L. Eugene Daily of the Eaton Laboratories, Norwich, N. Y. (Lot No. 1050).

[1] Dodd, M. C., and Stillman, W. B., *J. Pharm. and Exp. Therap.*, 1944, **82**, 11.

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[6] Shipley, E. R., and Dodd, M. C., *Surg., Gynec., and Obst.*, 1947, **84**, 366.

[7] Green, M. N., and Mudd, Stuart, PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 57.

TABLE I.

Effect of Furacin on the Growth of H37Rv Tubercle Bacilli in Four Different Types of Fluid Medium.

A. Proskauer and Beck's Synthetic Medium.

	Amt of growth in days				
Furacin Conc.	8	12	16	21	28
Control	+	+	+	2+	2+
1:80×1000	+	+	+	2+	2+
1:40 ”	0	±	+	+	2+
1:20 ”	0	0	0	0	0
1:10 ”	0	0	0	0	0
1: 5 ”	0	0	0	0	0

B. Proskauer and Beck's with 10% Human Serum.

	Amt of growth in days				
Furacin Conc.	4	8	12	16	22
Control	+	2+	3+	4+	4+
1:80×1000	±	2+	3+	3+	3+
1:40 ”	±	+	2+	2+	3+
1:20 ”	0	±	±	±	±
1:10 ”	0	±	±	±	±
1: 5 ”	0	0	0	0	0

C. Kirschner's Synthetic Medium.

	Amt of growth in days				
Furacin Conc.	8	12	16	21	28
Control	3+	3+	4+	4+	4+
1:80×1000	2+	2+	3+	4+	4+
1:40 ”	+	+	2+	2+	2+
1:20 ”	±	±	±	±	±
1:10 ”	0	±	±	±	±
1: 5 ”	0	0	0	0	0

D. Tween-albumin Liquid Medium.

	Amt of growth in days				
Furacin Conc.	4	8	12	16	22
Control	2+	4+	4+	4+	4+
1:80×1000	+	3+	4+	4+	4+
1:40 ”	±	2+	3+	4+	4+
1:20 ”	0	0	0	±	±
1:10 ”	0	0	0	0	0
1: 5 ”	0	0	0	0	0

Using an inoculum of 0.2 cc (twice the original amount), it was found that a 1:20,000 concentration of Furacin still inhibited growth of the organisms. The H37 Rv culture which had been made resistant to streptomycin exhibited the same sensitivity to Furacin as the original streptomycin-sensitive strain.

Fig. 1 illustrates the effect of different concentrations of Furacin on the turbidity produced in Tween-albumin medium by the H37 Rv culture, as determined in the Klett-Summerson colorimeter. The technic was the same as that described previously for streptomycin.[8] Here again, it will be observed that Furacin in a 1:40,000 dilution produced slight inhibition of growth of the organisms, whereas the drug in a 1:20,000 dilution prevented the development of turbidity for 11 days.

Furacin exerts a bactericidal action on tubercle bacilli, as well as a growth-inhibiting action. This was illustrated by inoculating 0.5 cc of a culture of H37 Rv into 25 cc of Tween-albumin medium in a small flask, which was then incubated at 37°C. Similar flasks were set up, containing Furacin in dilutions of 1:40,000, 1:20,000, 1:10,000 and 1:5,000. At various intervals, samples were removed, appropriately diluted with Tween-albumin medium, and 0.1 cc amounts spread evenly over the surface of a solid egg-yolk, potato medium contained in flat glass tubes. Each dilution was planted in triplicate. Colony counts were made after incubation of the slants (in a horizontal position) for 30 days.

Fig. 2 illustrates the results of the experiment. Furacin in a concentration of 1:20,000 produced only a small decrease in the number of viable cells after 7 days, but in a concentration of 1:5,000 the drug had rendered non-viable the vast majority of tubercle bacilli by the 7th day.

In contrast to the bactericidal effect of Furacin on multiplying bacilli, there was no killing action whatsoever when the flasks were kept in the refrigerator at 5°C.

Tubercle bacilli of the H37 Rv strain could not be made resistant to the action of Furacin in the test tube. This culture was inoculated into a series of tubes of Tween-albumin medium containing the following concentrations of the drug: 1:80,000, 1:40,000, 1:20,000, 1:10,000, and 1:5000. After 10 to 12 days' incubation the medium in the tube containing the highest concentration of Furacin which allowed growth was used to inoculate a similar series of Furacin dilutions. At the end of

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[8] Wolinsky, E., and Steenken, W., Jr., *Am. Rev. Tuberc.*, 1947, **55**, 281.

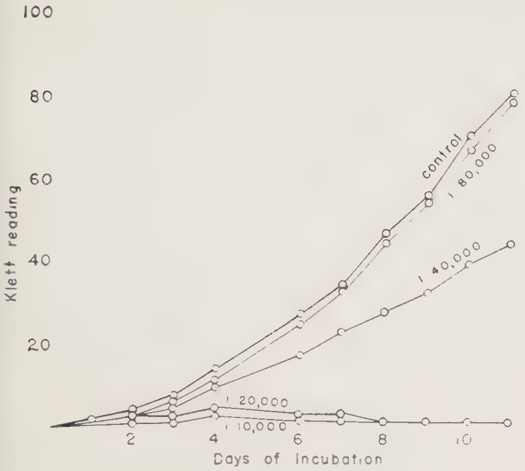

FIG. 1.
Effect of Furacin on growth of H37 "Rv" in Tween albumin medium.

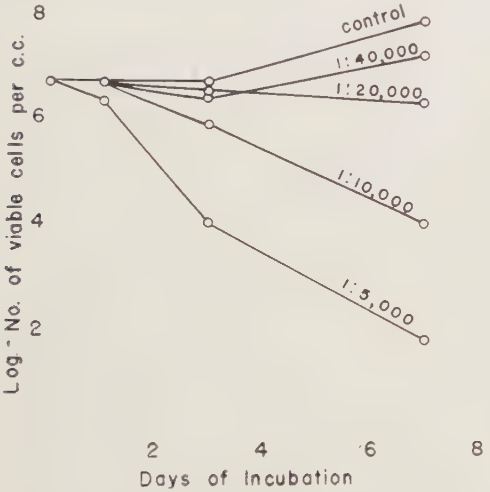

FIG. 2.
Bactericidal action of Furacin on H37 "Rv" in Tween albumin medium.

16 such transfers, growth of the culture was still inhibited by a 1:20,000 dilution of the drug.

The possible inhibitory effect of Furacin on the development of streptomycin resistance was tested *in vitro*. A sufficient amount of Furacin to make a final concentration of 1:50,000 was added to Tween-albumin liquid medium containing graded concentrations of streptomycin. Serial transfers of the H37 Rv culture were made to progressively higher concentrations of streptomycin in the usual manner, and the rate at which resistance developed was compared with a similarly handled culture in the absence of Furacin. It was found that the presence of Furacin in the medium delayed, but did not prevent, the development of streptomycin resistance by the tubercle bacilli.

In Vivo Tests in Guinea Pigs. Before initiating drug therapy, the toxicity of Furacin for guinea pigs was investigated. The drug was suspended in 10% gum acacia in a concentration of 150 mg per cc, and fed to normal pigs (average weight, 500 g) by means of a tuberculin syringe fitted with a blunt needle. It was found that 150 mg once a day, or 75 mg twice a day, killed all the animals used in the test within a period of 5 days. Guinea pigs fed 75 mg per day, either in 1 dose or 2, appeared very ill by the 10th day, and it was obvious that all the animals would have died shortly. The dosage was reduced to 37.5 mg once a day for 10 days. This treatment period was followed by a rest period of 10 days, after which time the drug was resumed. Three animals survived 28, 32, and 44 days, respectively, before succumbing to the toxic effects of the drug.

The major experiment was then begun. Thirty-five guinea pigs were infected with the human type H37 Rv culture. Each animal received 0.05 mg (dry weight) subcutaneously in the inguinal region. They were of mixed sexes and weighed between 600 and 900 g. Ten were kept as untreated controls, and 25 were started on Furacin treatment 6 days after infection.

The drug was suspended in 10% gum acacia in concentration of 75 mg per cc. Each treated animal was fed 18.75 mg at 12-hour intervals. After 11 days of treatment, several animals appeared listless and refused to eat. By the 13th day, 6 guinea pigs had died, and treatment was suspended for 17 days. Despite this rest period, another 5 animals died during the next 6 days, evidently of Furacin toxicity.

When Furacin was resumed 17 days later, the pigs had recovered sufficiently to appear healthy and to have good appetites again. The drug was then given in dosage of 18.75 mg once a day until the experiment was terminated by sacrificing all survivors 70

TABLE II.
Results in Guinea Pig Tuberculosis Treated with Furacin.

G. pig No.	Fate	Days of infection	Days of* treatment	Amt of tuberculosis grossly at autopsy
Controls				
1	Died	19	0	6+
2	”	63	0	9.5+
3	”	66	0	7.5+
4	”	67	0	14+
5	Killed	71	0	10+
6	”	”	0	9+
7	”	”	0	11+
8	”	”	0	8+
9	”	”	0	9+
10	”	”	0	7.5+
				Avg‡ 9.1+
Treated				
1	Died	13	7	0
2	”	17	11	1+
3	”	18	12	4+
4	”	”	”	5.5+
5	”	19	13	3+
6	”	”	”	3+
7	”	21	15	4.5+
8	”	22	16	3+
9	”	23	17	3+
10	”	25	19	4+
11	”	”	”	4.5+
12	”	53	47	12+
13	”	56	50	6+
14	”	61	55	7+
15	”	67	61	7+
16	Killed	70	64	10+
17	”	”	”	6.5+
18	”	”	”	7+
19	”	”	”	12+
20	”	”	”	10.5+
21	”	”	”	11+
22	**	”	”	10+
23	”	”	”	10+
24	”	”	”	9.5+
25	”	”	”	9.5+
				Avg‡ 9.6+

* Including rest period of 17 days without Furacin after the first 13 days of treatment.
‡ Average of those animals killed at the termination of the experiment.

days after infection, or 64 days after Furacin treatment was begun.

The results of this experiment are shown in Table II. The amount of tuberculous disease in the spleen, liver, lungs, and lymph nodes of each pig was graded from zero to 4+, according to the gross appearance. Thus, the maximum disease for an animal was 16+. The average degree of tuberculosis of the 6 controls which survived the full period of 70 days was 9.1+, whereas the corresponding figure for the 10 Furacin-treated animals which were killed at the termination of the experiment was 9.6+ It was thus demonstrated that Furacin, in the comparatively low dosage necessary because of the evident toxicity for guinea pigs, had no tuberculostatic effect on this infection produced by H37 Rv organisms.

Discussion. The experiments herein described reveal that Furacin is a compound which exerts definite bacteriostatic and bactericidal action on virulent human tubercle bacilli in the test tube; upon the progression of a tuberculous infection in guinea pigs, however, the drug appears to have no inhibitory effect.

There are some possible explanations for this failure *in vivo*. Furacin is relatively insoluble and must be administered as a suspension of large particles which tend to settle out easily. It is not known how much is absorbed or what happens to the compound in the body. No Furacin can be demonstrated in the blood of animals to whom the drug has been given, and only a very small amount in the urine.[9] It is not known how much of the drug is converted to an inactive compound in the body of the guinea pig.

The best chemotherapeutic results in mice infected with Staphylococci, Streptococci, and Salmonella organisms were obtained by feeding 150 to 200 mg per kg daily for 3 days.[5] It was not possible to administer this amount of Furacin to guinea pigs without fatal results, and it was necessary to decrease the dose to about 35 mg per kg daily, which seemed to be the maximum tolerated dose for prolonged therapy. This relatively small amount of Furacin was probably not sufficient to produce a bacteriostatic or bactericidal level in the body of the guinea pig.

It was observed that the toxic effects of Furacin continued to be manifest for many days after the administration of the drug was stopped. On the regimen of 18.75 mg per day, the guinea pigs appeared healthy for 10 days, but then almost all began to show loss of appetite, listlessness, and loss of weight. Deaths started to occur on the 11th day. Even after the drug was withdrawn, some animals continued to decline and die for the next 5 or 6 days, before improvement in the appearance of the group was noted.

Conclusions. 1. Furacin exerts a bacteriostatic and bactericidal action on virulent human tubercle bacilli *in vitro*. In a concentration of 1:20,000 (5 mg %) it prevented growth for 22 days in 4 different types of fluid media. A concentration of 1:5000 (20 mg%) was required for bactericidal effect.

2. Resistance to Furacin was not evident after many transfers of the H37 Rv culture in drug-containing medium.

3. The combination of Furacin and streptomycin in Tween-albumin liquid medium delayed, but did not prevent, the development of streptomycin-resistance by the H37 Rv tubercle bacillus.

4. Furacin, in the maximum tolerated dose of about 35 mg per kg daily for 64 days, did not appreciably affect the course of tuberculosis produced in guinea pigs by the human type H37 Rv culture.

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[9] Unpublished results, Eaton Research Laboratories.

16967

Effects of Adding Carbon Dioxide to Inspired Oxygen on Tolerance to High Altitudes.*

A. B. OTIS, H. RAHN, AND L. E. CHADWICK.
From the Department of Physiology and Vital Economics, The University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.

When CO_2 is added to the inspired air at altitudes around 20,000 feet or to low oxygen mixtures, there is an improvement in the physiological condition which is due to an increase in the alveolar pO_2.[1-4] There is no general agreement, however, on the question

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* Work done under contract recommended by Committee on Medical Research between the Office of Scientific Research and Development and the University of Rochester, and under contract with Air Materiel Command, Wright Field.

[1] Fenn, W. O., Rahn, H., and Otis, A. B., *Am. J. Physiol.*, 1946, **146**, 637.

[2] Rahn, H., and Otis, A. B., *Am. J. Physiol.*, 1947, **150**, 202.

[3] Gray, J. S., A.A.F. School of Aviation Medicine, Randolph Field, Project Report 310, August, 1944.

[4] Gibbs, F. A., Gibbs, E. L., Lennox, W. G., and Nims, L. F., *J. Av. Med.*, 1943, **14**, 250.

of whether any advantage is gained by adding CO_2 to pure oxygen at high altitudes. For example, Humm *et al.*[5] and Garasenko[6] seem to find favorable effects. On the other hand, Johnson *et al.*[7] and Himwich *et al.*[8] are unable to find any benefit. The data reported below confirm the latter point of view.

Experiments on Mice. Four series of mice maintained on a stock diet were exposed in an evacuated chamber (a vacuum desiccator) to simulated altitudes in excess of 40,000 feet in various gas mixtures. Before the experiments were begun, the mice were conditioned individually to sit on a horizontal cross bar, ½ inch in diameter, which extended across the experimental chamber 3 inches above the floor. The latter consisted of a double metal grill which was connected to the secondary coil of an inductorium. When a mouse was on the grill, the operator could shock him at will by depressing a key in the primary circuit. After a few trials, the mice learned to seek safety on the cross bar, and would voluntarily take position there whenever they were put into the chamber. It was thus possible to use as an end point in the experiments the altitude at which a mouse was no longer able to maintain himself on the cross bar. In this way there was established a fairly definite criterion which did not depend on the judgment of the experimenter or place too great a strain on the endurance of the mice. After proper conditioning each mouse was exposed to altitude during a number of runs in each of the several gas mixtures.

The procedure was varied in the 4 series, as follows:

Series A. Flushout with 100% oxygen at ground level for 3 minutes; ascent to 30,000 feet in 1½ min.; flushout with experimental mixture for 5 min.

Ascent to 40,000 feet in ½ min.; stay 5 min.

Further ascent in 2000-foot stages, with 5 min. at each altitude, until ceiling was reached.

Temperature varied from 22-25°C.

Three mixtures were used: 100% oxygen (April 29 to May 5), 21.7% CO_2 in O_2 (May 8 to May 20); 20% N in O_2 (May 28 to June 13).

Series B. Flushout with 100% oxygen at ground level for 2 min.; ascent to 30,000 feet in 1½ min.; flushout with test mixture for 3 min.; continuous ascent to ceiling altitude at rate of *ca.* 6,000 ft. per min.; run in constant temperature room at 20-21°C. Three mixtures were used: 100% O_2, and 21.7% CO_2 in oxygen, on alternate days between July 10 and July 28; 10.5% CO_2 in oxygen (August 8 to August 15).

Series C. Procedure as in Series B, except that the chamber was flushed out with the test mixture at 30,000 ft. for 10 min. Two mixtures were used: 10.5% CO_2 in oxygen, and 100% oxygen, on alternate days between August 15 and August 28.

Series D. Same procedure as in Series A, except that temperature was between 20-21°C. Two mixtures were used in random succession: 10.5% CO_2 in oxygen and 100% oxygen.

In a given series, the same mice were used for all the different conditions tested. Some mice were used in more than one series. Each mouse was exposed no oftener than once a day, and usually less frequently. Only one mouse was tested in each run.

The volume of the experimental chamber was approximately 5 liters. Gas flow through the chamber was continuous whenever the altitude was stationary, and amounted to about 9 liters per minute at ground level, 2.5 liters (measured at 760 mm Hg) at 30,000 feet, 1.5 liters (at 760 mm Hg) at 40,000 feet, and 1.0 liter (at 760 mm Hg) at 50,000 feet. Inflow of gas was stopped in order to make an ascent. Altitudes were read with a mercury manometer. In the later experiments, the circuit was checked frequently for leaks, which were found to be negligible.

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[5] Humm, F. D., Liberman, A. M., and Nims, L. F., C.M.R. Report No. 335, July 18, 1944.

[6] Garasenko, V. M., *Am. Rev. Sov. Med.*, 1945, **2**, 119.

[7] Johnson, A. E., Eckman, M., Rumsey, C., and Barach, A. L., *J. Av. Med.*, 1942, **13**, 130.

[8] Himwich, H., Fazekas, J., Herrlich, H., Johnson, A. E., and Barach, A. L., *J. Av. Med.*, 1942, **13**, 177.

TABLE I.

Series	Gas mixture	Mean ceiling altitude, 10^3 ft	Standard deviation, 10^3 ft	S.D. of mean, 10^3 ft	No. of mouse runs	No. of mice
A	100% O_2	50.3	2.1	.33	41	6
	21.7% CO_2	50.9	2.4	.39	38	6
	20% N_2	49.5	2.1	.36	35	6
B	100% O_2	48.6	1.3	.19	50	15
	10.5% CO_2	47.4	1.6	.24	45	15
	21.7% CO_2	46.8	1.6	.22	53	15
C	100% O_2	48.6	1.3	.24	28	15
	10.5% CO_2	48.4	1.1	.19	29	15
D	100% O_2	53.4	2.5	.43	34	11
	10.5% CO_2	51.3	2.5	.42	36	11

TABLE II.
Summary of Measurements on Human Subjects Breathing 100% O_2 and 90% O_2-10% CO_2 at 40,000 Feet.

Subject	Min. vol., liters/min. B.T.P.S.		Oximeter, % saturation		Alveolar pCO_2, mm Hg	
	100% O_2	90% O_2-10% CO_2	100% O_2	90% O_2-10% CO_2	100% O_2	90% O_2-10% CO_2
R	14.9	18.2	92.6	91.8	31.9	34.9
S	17.5	21.1	90.6	90.5	37.2	38.1
H	10.3	12.2	92.1	91.8	37.8	37.8
Mean	14.2	17.2	91.8	91.4	35.6	36.9
Δ		+3.0		—0.4		+1.3

Results. Average results for each series are presented in Table I. In none of the series was there any significant increase in altitude ceiling as a result of addition of carbon dioxide to the experimental mixture.

In series A, the substitution of 20% nitrogen for oxygen resulted in a slight but probably real decrease in ceiling.

In series B, the performance was definitely worse in both carbon dioxide mixtures than in 100% oxygen, and probably worse in the higher than in the lower concentration of carbon dioxide. On the other hand, in series C where the mice spent 10 minutes at 30,000 feet in the experimental mixture, instead of only 3 minutes as in series B, approximately the same altitudes were reached in 10.5% carbon dioxide as in pure oxygen. In series D, which resembles series A in that 5-minute stops were made at 2,000-foot intervals above 40,000 feet, performance was definitely better in 100% oxygen.

In view of these findings, it may be concluded definitely that mice gain no benefit, in terms of altitude ceiling, from the substitution of carbon dioxide for oxygen with the patterns employed in these experiments. In some cases, a definite harmful effect (decreased ceiling with added carbon dioxide) has been demonstrated.

Differences in the average ceiling altitude attained in a given gas mixture in the several series may be due to:

(1) Differences in altitude tolerance of individual mice; (2) Slight temperature difference between series A and the other series;

(3) Variations in the adaptation of circulatory and other mechanisms resulting from different patterns (rates) of ascent.[9]

Experiments on Human Subjects. Each of 3 male subjects was taken to a simulated altitude of 40,000 feet wearing an ear oximeter (Millikan compensated circuit) and inspiring 100% oxygen through a gas meter. Measurements of the ventilation and oximeter readings were made during a 15-minute period, and near the end of this interval an end-expiratory alveolar sample was secured. The inspired gas was then changed to a mixture of 10% CO_2 and 90% O_2, and measurements were continued for another 15 minutes at the end of which another alveolar sample was taken.

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[9] Hiestand, W. A., and Miller, H. R., *Am. J. Physiol.*, 1944, **142**, 310.

Results are summarized in Table II. Substitution of the CO_2-O_2 mixture for pure oxygen produced a rise of 1.3 mm in the alveolar pCO_2 (with lowering of the alveolar pO_2 by the same amount) and a drop of 0.4% in the saturation as determined from the oximeter. The change in % saturation is within the error of the instrument, but the expected change for the change in alveolar gas tensions is about 1%. The minute volume of ventilation was increased by 3 liters per minute or about 21%. These data indicate that no favorable physiological changes occurred from the addition of 10% CO_2 (9.4 mm) to the inspired gas at 40,000 feet. One cannot argue from this that it would not be advantageous to add CO_2 under some conditions, but it must be kept in mind that any rise in alveolar pCO_2 is always accompanied by an equivalent drop in alveolar pO_2 when nitrogen is absent. Probably the only condition in which substitution of a CO_2-O_2 mixture for pure O_2 might be advantageous is that of severe hypocapnia accompanied by a relatively mild hypoxia. In such a case the improvement due to the decreased hypocapnia might be greater than the impairment due to the increased anoxia.[10]

Summary. 1. The addition of 10.5% or 21.7% carbon dioxide to oxygen does not improve the altitude ceiling of mice.

2. Mice taken to altitude in 100% oxygen, with continuous ascent from 30,000 feet at a rate of about 6,000 feet per minute, collapse at a lower ceiling than mice which ascend from 40,000 feet in 2,000-foot steps with 5-minute stops at each stage.

3. In experiments on human subjects no favorable physiological effects were observed when 10% CO_2 was added to the inspired oxygen at 40,000 ft.

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[10] Otis, A. B., Rahn, H., Epstein, M. A., and Fenn, W. O., *Am. J. Physiol.*, 1946, **146**, 207.

16968

Simplified Indirect Complement-Fixation Test Applied to Newcastle Disease Immune Avian Serum.

DON M. WOLFE, LOTTIE KORNFELD, AND FLOYD S. MARKHAM.
(Introduced by Herald R. Cox.)

From the Section of Viral and Rickettsial Research, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

The investigation in this laboratory of an avian disease presented a problem of accurate specific diagnosis. Because antigens suitable for the performance of the customary agglutination and precipitin tests were not available, attention was turned to the complement-fixation reaction. The development of the *indirect* method by Rice[1,2,3] made this approach more feasible since this technic apparently overcomes the difficulties which have prevented the use of the regular, or *direct*, method on avian serums.[4]

It also seemed worthwhile to attempt to simplify the *indirect* technic by using the common "serum or antigen-dilution" method and the observation of 100% hemolysis rather than the more detailed quantitative method for the standardization of all test com-

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[1] Rice, C. E., *J. Immunol.*, 1947, **59**, 365.

[2] Rice, C. E., *Canadian J. Comp. Med.*, 1948, **12**, 130.

[3] Rice, C. E., *J. Immunol.*, 1948, **60**, 11.

[4] Rice, C. E., *Canadian J. Comp. Med.*, 1947, **11**, 236.

ponents.[5]

The work reported herein describes a study of the Newcastle disease (NCD) virus and immune serum which served as a convenient working model for the evaluation of such a test with avian serum. The hemagglutination-inhibition test was conducted simultaneously to provide a rough estimate of the reliability of the *indirect* complement-fixation results. Since chickens are known to possess an excellent capacity for antibody production, and their serums have been used for the study of various infections (influenza, psittacosis, etc.), a description of a simplified *indirect* complement-fixation test may prove of use to other investigators.

Materials and Methods. NCD immune avian serums. Serum samples were collected from chickens previously vaccinated against, or recovered from, Newcastle disease. *NCD immune guinea pig serum.* A group of guinea pigs received weekly subcutaneous injections of 0.5 ml each for 4 consecutive weeks of the antigen described below, and were bled 1 week after the final inoculation. Before pooling, the individual serum specimens were tested for complement-fixing activity by the *direct* method and only those showing titers of 1:128 or higher were included in the pool. All serum specimens were stored at 4°C.

NCD antigen. Allantoic fluids harvested from infected 11-day-old chick embryos were pooled and the virus concentrated by Sharples centrifugation. The sedimented virus was washed with 0.1 *M* phosphate buffer (pH 7.0) and resuspended in one-half the original volume of buffered saline solution[6] containing 0.05% formalin. A single lot of antigen was used in the complement-fixation and hemagglutination-inhibition tests described below.

Indirect Complement-Fixation Test. Prior to use, all serums were inactivated at 56°C for 30 minutes. Physiological salt solution was added as a diluent for all test components.

In the antigen-immune guinea pig serum titrations and in the tests proper, antigen, immune chicken serum and complement were incubated at 4 to 6°C for 2 hours. Immune guinea pig serum was then added and the tubes returned to the cold room for an additional 16- to 18-hour period (overnight). Sensitized sheep cells were added and hemolytic readings were made after an incubation period of 30 minutes at 37°C. All test components were employed in 0.2 ml amounts, thus making the total volume of 1.2 ml in all titrations and tests.

Sheep Cells. Defibrinated sheep erythrocytes were washed by centrifugation in saline solution, standardized spectrophotometrically, and adjusted to a concentration of 2%.[7]

Amboceptor. Commercial antisheep hemolysin was titrated in the usual manner and 2 units were used in all titrations and tests. A sensitized sheep cell suspension was prepared by mixing equal volumes of 2% cells and amboceptor and allowing the mixture to stand for 15 minutes at room temperature prior to use.

Complement. Serums of normal guinea pigs were pooled, 2 ml amounts were sealed in pyrex ampoules, and stored in the CO_2 icebox. Complement activity remained constant for at least 3 weeks.

For titration, amounts ranging from 0.08 to 0.22 ml (in increments of 0.02 ml) of a 1:30 dilution of complement were mixed with 0.2 ml of antigen (containing the optimal reacting amount) and with saline solution to bring the volumes to 0.8 ml. These mixtures were incubated for 1 hour at 37°C. Four-tenths ml sensitized cells were then added and hemolytic readings were made at the end of an incubation period of 1 hour at 37°C. The smallest amount of complement giving complete hemolysis was taken as 1 unit.

Preliminary titrations of NCD antigen and homologous immune guinea pig serum were carried out by the *direct* complement-fixation method using 2 units of complement. However, in the *indirect* tests, involving the determination of optimal amounts of antigen and immune guinea pig serum to be used in the titration of an immune chicken serum, it was found that 1.5 units of complement gave more uniform and consistent results than the larger quantity. This amount is approximately equivalent to the three 50% units employed by Rice.[2,3]

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[5] Wadsworth, A. B., Standard Methods of the Division of Laboratories and Research, New York State Department of Health, Albany, N. Y., Williams and Wilkins, Baltimore, Md., 1947, 3rd ed., pp. 361-465.

[6] Eagle, H., The Diagnosis of Syphilis, Mosby and Co., St. Louis, Mo., 1938.

[7] Kent, J. F., Bukantz, S. C., and Rein, C. R., *J. Immunol.*, 1946, **53**, 37.

Titration of Antigen. A preliminary antigen titer of 1:8 was obtained by the simultaneous "cross titration" of both antigen and immune guinea pig serum by the *direct* method. It will be seen that this titration furnished a fairly reliable basic titer for use in the *indirect* method. The titration by the latter method is described in the following section.

Titration of Immune Guinea Pig Serum. That the antigen and known immune serum (guinea pig) be present in proportions which would permit maximal inhibition of fixation by the test serum (chicken), has been stressed by Rice.[1,2] A simplified procedure for the titration of these components was developed by simultaneously testing varying amounts of antigen and immune guinea pig serum in the presence of varying amounts of a known immune chicken serum, as illustrated in Table I.

TABLE I.
A Protocol Demonstrating the Method for Determining the Optimal Proportions of Antigen and Immune Guinea Pig Serum to Be Used in the Indirect Complement Fixation Procedure.

Dilutions of antigen	Dilutions of immune guinea pig serum	Dilutions of immune chicken serum 1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	Control*
1:8	1:70	—	—	—	—	++	++++	++++	++++	++++
	1:80	—	—	—	—	±	+++	++++	++++	++++
	1:90	—	—	—	—	—	+++	++++	++++	++++
	1:100	—	—	—	—	—	—	++	++++	++++
	1:110	—	—	—	—	—	+	+	+++	++
	1:120	—	—	—	—	—	—	+	+	++
	1:130	—	—	—	—	—	±	+	++	+++
1:16	1:20	—	—	—	—	+	++	++++	++++	++++
	1:30	—	—	—	—	—	++	+++	+++	++++
	1:40	—	—	—	—	—	+	+	+++	+++
	1:50	—	—	—	—	—	—	+	+++	+++
	1:60	—	—	—	—	—	—	++	++	++

* Immune chicken serum replaced by saline solution.

The amounts of antigen and immune guinea pig serum giving complete inhibition of hemolysis in the control tube (*i.e.*, in the *absence* of immune chicken serum), and complete hemolysis in the presence of the smallest quantity of immune chicken serum, were selected as the optimal amounts to be used in subsequent tests. In this case an antigen dilution of 1:8 and an immune guinea pig serum dilution of 1:100 were found to be the properly reacting quantities of these components.

Test. The test technic was similar to that described by Rice[2] in which serial twofold dilutions of the suspected immune chicken serum were prepared in triplicate. Antigen was added to sets No. 1 and No. 2, saline solution to set No. 3. All tubes then received complement. After the preliminary cold incubation, immune guinea pig serum was added to set No. 1 and saline solution to sets No. 2

TABLE II.
Results Obtained by the Indirect Complement-Fixation Test of 59 Newcastle Disease Immune Chicken Serums Compared with Hemagglutination-Inhibition Test.*

Indirect complement fixation test	Hemagglutination-inhibition test									
	0	1:16	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096	1:8192
0	3	1		2		1				
1:2			2							
1:8			2	3	4	2				
1:16			1	2	6	2				
1:32				1	2	4	2			
1:64				1	1	1	1			
1:128					1	2	1			
1:256					3		1	3	1	
1:512								1		
1:2048									1	1

* The numbers in the body of the table refer to the number of serums giving the endpoint titers indicated.

and No. 3. Thus, set No. 1 represented the *indirect* test, No. 2 served as the *direct* test, and No. 3 as the control for anticomplementary activity of the test serum. Additional controls were included daily for hemolytic activity of antigen, immune guinea pig serum, and antigen plus immune guinea pig serum, as well as the titration of a standard NCD immune chicken serum.

The titer of the test serum was expressed in terms of the highest dilution which inhibited fixation of complement by exhibiting ++ hemolysis or more.

Hemagglutination-Inhibition Test. Titration of Antigen. Serial twofold dilutions of virus were prepared in buffered saline solution, pH 7.1.[6] To 0.5 ml amounts of each dilution was added an equal volume of 0.25% chicken red blood cells and the mixture incubated at room temperature for 1 hour. The highest final dilution* of virus giving complete agglutination of the red cells was designated as 1 virus unit.

Test. On the basis of the above titration, a dilution of antigen containing 16 units per ml was made and used as diluent in the preparation of twofold dilutions of the test serum. To 0.5 ml amounts of the serum-virus mixtures was added an equal volume of 0.25% chicken red blood cells. Tests were read after an incubation period of 1 hour at room temperature. The endpoint of serum-inhibition was that final dilution* of serum which completely inhibited agglutination, as indicated by the flowing of the red cell deposit to a degree comparable to the red cell saline control when the test tube rack was tilted and held at approximately 70 degrees from horizontal.

Discussion of Results. The data presented in Table II show that a good, general agreement exists between the titers obtained by the *indirect* complement-fixation and by the hemagglutination-inhibition tests. No anticomplimentary serums were encountered. Four serums were negative when examined by the *indirect* complement-fixation test, yet gave positive results ranging from 1:16 to 1:512 with the other method. Nevertheless, those serums which exhibited high titers by one method also did with the other. The same holds true for specimens of intermediate and low antibody titer.

No effort was made to determine whether or not the two tests were measuring the same serum component. It is quite possible that different antibodies are involved, hence too much emphasis should not be placed on the observed agreement of test results until further information has been obtained.

However, the results of this study suggest the possibility that the described simplified *indirect* complement-fixation test should provide a useful tool in investigations involving the examination of serums of certain species in which the *direct* complement-fixation procedures are not feasible.

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* This term refers to the dilution of the original amount of a particular component in terms of its subsequent dilution by the addition of other components. Thus, an *initial* serum dilution of 1:8, in this case, has a *final* dilution of 1:16.

Summary. 1. A simplified procedure for the *indirect* complement-fixation test is described.

2. The serums of 59 Newcastle disease immune chickens were tested by the simplified *indirect* complement-fixation and by the hemagglutination-inhibition tests.

3. Good general agreement was observed between the titers obtained by the two methods.

16969

Preparation and Amino Acid Composition of Salmin and Clupein.

RICHARD J. BLOCK, DIANA BOLLING, HERMAN GERSHON, AND HERBERT A. SOBER.
From the Department of Physiology and Biochemistry, New York Medical College, New York.

Salmin, on acid hydrolysis, was shown to yield arginine, proline, serine, valine, alanine, and isoleucine.[1] This observation was confirmed and extended by Tristram[2] who also identified glycine. This paper confirms the presence of glycine in salmin and also indicates that clupein, from *Clupea pallasii*, differs from salmin in that it contains threonine but no glycine.

1. *Preparation.* The procedure to be described gives approximately 10 times the yield of protamine from frozen milt than was obtained by any of the older methods.[3] One thousand grams of frozen milt are thawed and rinsed with cold tap water, mixed with an equal volume of a 1% solution of citric acid, finely ground, and 2.5 liters of 1% of citric acid are added. After standing in the refrigerator over night, the insoluble material is removed by centrifugation and the residue is washed with citric acid. This precipitate is suspended in 4 liters of 0.2 *N* HCl for 18-24 hours at room temperature with occasional mixing. The precipitate is removed and washed with 0.2 *N* HCl. The combined filtrate and washings are heated to boiling and adjusted to pH 7-8 with dilute ammonia. The resulting precipitate is removed by centrifugation and ammonia is added to the filtrate, at 30°C, to bring the pH to 9.4-9.6. The protein precipitate is removed by filtration. The contaminating proteins may also be removed by precipitation with 14% ammonia at room temperature at pH 10.4-10.6. In contrast to the first method, the histones (?) so prepared are completely soluble in 0.2 *N* HCl.

The filtrate is cooled to 12-15°C. Aliquots of the cold solution are removed and graded quantities of freshly prepared 33% of HPO_3 are added. The oily precipitates of protamine metaphosphate are thrown down by centrifuging and are washed with cold water. The quantity of HPO_3 required for optimal precipitation is thus ascertained.

The calculated quantity of 33% HPO_3 is then added slowly with stirring to the main protamine solution and the resulting oil is removed and washed with cold water until the washings are free from ammonia. The protamine metaphosphate precipitate is dissolved in a minimum quantity of hot *N* sulfuric acid. The solution is boiled until a small aliquot no longer gives a white cloud when cooled below 5°C, *i.e.*, when the metaphosphate has been completely converted to the sulfate but no longer. Protamine sulfate is precipitated and dried by the addition of alcohol or acetone. The yield is 2.0-2.5% of the weight of the original milt.

More than 30 preparations of salmin sulfate or hydrochloride and 8 preparations of clupein sulfate have been prepared from samples of

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[1] Block, R. J., and Bolling, D., *Arch. Biochem.*, 1945, **6**, 419.

[2] Tristram, G. R., *Nature,* 1947, **160**, 637.

[3] Kossel, A., Protamine und Histone; Leipsig, Wien, 1929.

TABLE I.
Amino Acids in Salmin and Clupein (free base).

	Salmin				Clupein	
Amino acid	Block and Bolling[1] and Authors %	Kossel and Dakin (*cf.* [3]) %	Taylor[12] %	Tristram[2] %	Authors %	Kossel[3] %
Arginine	88.4	87.4	91.7	85.2	87.3*	88.0
,,					86.8†	
Proline	7.9	11.0	10.8	5.8	8.2†	
,,	8.6†					
Serine	7.0	7.8	8.7	9.1	3.4	
Valine	4.1	4.3	5.3	3.1	3.6	
Alanine	1.5			1.1	4.7	
Isoleucine	1.2			1.6	1.0	
Leucine	0†				0†	
Glycine	3.3			2.9	0	
Threonine	0				1.9	
Nitrogen	31.52	31.52			31.68	

* Kossel flavianate method.
† Microbiological method.

salmon or herring milt obtained through the kindness of Mr. Dudley Cole, Victoria, B.C., during the past 4 years. The protamine sulfate preparations were devoid of contaminating material as evidenced by negative Molisch, Millon, Pauly, ninhydrin, and phosphorus tests. (*cf.*[1,4]). The purity of each preparation was further confirmed by the use of two-dimensional paper chromatograms.[5]

2. *Amino Acid Analysis.* Arginine was determined by precipitation as the monoflavianate (*cf.*[4]) and microbiologically.[6] Proline was estimated by Guest's pyrrole method (*cf.* [4]) and by the microbiological procedures of Henderson and Snell[7] or of Dunn *et al.*[8] Serine and threonine were determined by periodate oxidation (*cf.* [4]). Glycine and alanine were determined by oxidation with ninhydrin to HCHO and CH_3CHO respectively (*cf.* [4,9,10]) Leucine, isoleucine, and valine were estimated by microbiological methods[4,11,12]. Leucine does not appear to be present in either of these protamines.

Results and Discussion. Our analytical results, as well as those of Kossel and Dakin (*cf.* [3]), Taylor,[13] and Tristram,[2] are given in Table I.

In contrast to the results of Felix and Mager,[14] there is no evidence for the presence of hydroxyproline in our samples of clupein. Because salmin contains no demonstrable amino or imino nitrogen or free carboxyl groups, Frankel-Conrat and Olcott[15] suggested that salmin lacks an end-group and that it may have a cyclic structure similar to those suggested for gramicidin and tyrocidine. Waldschmidt-Leitz *et al.*[16] reported that the imino group of proline is one terminal group of salmin and the carboxyl of arginine the other. Tristram[2] mentions unpublished results of Porter using the dinitrofluorobenzene method as strong evidence that proline is the end-group of salmin. This may account for the absence of the ninhydrin color of these protamines.

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[4] Block, R. J., and Bolling, D., The Amino Acid Composition of Proteins and Foods. Analytical Methods and Results. C. C. Thomas, Springfield, Ill., 1945.

[5] Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, **38**, 224.

[6] Stokes, J. L., *et al.*, *J. Biol. Chem.*, 1945, **160**, 35.

[7] Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, 1948, **172**, 15.

[8] Dunn, M. S., Shankman, S., Camien, M. N., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, 1944, **156**, 703.

[9] Alexander, B., Landwehr, G., and Seligman, A. M., *J. Biol. Chem.*, 1945, **160**, 51.

[10] Alexander, B., and Seligman, A. M., *J. Biol. Chem.*, 1945, **159**, 9.

[11] Shankman, S., *J. Biol. Chem.*, 1943, **150**, 305.

[12] McMahan, J. R., and Snell, E. E., *J. Biol. Chem.*, 1944, **152**, 83.

[13] Taylor, A. E., *J. Biol. Chem.*, 1908-09, **5**, 389.

[14] Felix, K., and Mager, A., *Zeit. physiol. Chem.*, 1937, **249**, 112.

[15] Frankel-Conrat, H., and Olcott, H. S., *Fed. Proc.*, 1947, **6**, 253.

[16] Waldschmidt-Leitz, E., Ziegler, F., Schäffner, A., and Weil, L., *Z. physiol. Chem.*, 1931, **197**, 219.

The data obtained in this study indicate that the ratio of arginine to monoamino acids in both salmin and clupein is somewhat higher (*cf.* [1]) than the 2:1 ratio postulated by Kossel.[3]

The question of homogeniety of salmin was discussed in a previous publication.[1] A similar situation holds with respect to clupein. Since that time, numerous unsuccessful attempts to fractionate salmin and clupein have been made from 90% aqueous isopropanol at 90°C, 25°C, and 0°C, and by paper chromatography using water-saturated phenol, lutidine-water-alcohol, water alone, 0.1 N H_2SO_4 and other solvents. The dinitrophenyl derivatives of the protamines also failed to yield more than one spot on paper chromatograms using various solvents. Although these preparations may be chemically inhomogeneous, their overall physical properties must be rather similar due to the preponderance of arginine.

Summary. A simplified procedure for the preparation of salmin and clupein is described. The amino acid content of clupein as well as salmin has been estimated.

16970

Effect of Electronarcosis on Level of "Adrenalin-like" Compounds in Blood.

ESTHER BOGEN TIETZ AND A. VAN HARREVELD.

From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena, Calif.

A chemical method for the quantitative determination of adrenalin was devised by Whitehorn.[1] This procedure was modified by Shaw[2,3] who showed that the method is not specific for this compound, and that a number of substances related to adrenalin give the same reaction. Shaw's method was applied to blood by Tietz, Dornheggen and Goldman,[4] Raab,[5] Bloor,[6] and others. It became apparent that the material thus estimated is not adrenalin alone, but that other compounds determined by Shaw's procedure are present in the blood.

Previous observations showed that the level of these "adrenaline-like" compounds is increased in the blood of patients during insulin shock.[7] It was also found to be increased during electronarcosis and electroshock treatment in humans.[8] In the present paper the effect of electronarcosis on the level of these compounds was investigated experimentally.

Methods. The technic used for the estimation of adrenalin-like compounds in blood has been reported before.[7] In order to limit the total amount of blood taken from the animal the method was adapted in the following way to the use of about 2 cc per determination. Blood was taken from the jugular vein which had been exposed previously under local anesthesia. A little more than 2 cc of blood was drawn from the vein in an iced syringe and squirted immediately into a small tube cooled in ice. Of this, 2 cc was measured with an iced pipette in a test tube and mixed with 4 cc 10% trichloracetic acid. After 30 minutes 8 cc 5% trichloracetic acid was added and after inverting the tube twice the contents were filtered. The filtrate was kept in the ice box and 2 cc were used for each determination. The amount of adrenalin-like compounds was expressed as the adrenalin chloride equivalent (in gammas/100 cc of blood).

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[1] Whitehorn, C., *J. Biol. Chem.*, 1935, **108**, 633.

[2] Shaw, F. H., *Biochem. J.*, 1938, **32**, 19.

[3] Shaw, F. H., *Australian J. Exp. Biol. and Med. Sc.*, 1946, **24**, 53.

[4] Tietz, E. B., Dornheggen, H., and Goldman, D., *Endocrinology*, 1940, **26**, 641.

[5] Raab, W., *Endocrinology*, 1941, **28**, 325.

[6] Bloor, W. R., *J. Biol. Chem.*, 1939, **128**, ix.

[7] Tietz, E. B., and Birnbaum, S. M., *Am. J. Psych.*, 1942, **99**, 75.

[8] Tietz, E. B., unpublished data.

Electronarcosis was produced by applying a 60-cycle alternating current to the brain through electrodes placed on both sides of the head directly behind the eyes. Electronarcosis was started with a relatively high current 150-200 mA, which was continued for 30 seconds. The current was then dropped to 25-40 mA after which respiration was reestablished. This current was increased or decreased as needed to maintain a quiet electronarcosis.[9] The total duration of electronarcosis in the present experiments was limited to 5 minutes. Rabbits were used exclusively.

Results. In order to establish a control two blood samples were taken with a 10-minute interval before electronarcosis. In 33 experiments the mean of the level of adrenalin-like compounds in the first of these samples was 21 gamma/100 cc; the individual values varying between 17 and 25 gamma/100 cc. The difference between the two control values supplies information about the variability of the determination and about changes in the concentration of the adrenalin-like compounds during a 10-minute period. The mean difference in these 33 experiments was 0.03 gamma/cc, the largest difference was 3 gamma/cc. Using a different modification of the Whitehorn procedure, Kobro[10] obtained comparable values for the adrenalin-like compounds in rabbit's blood.

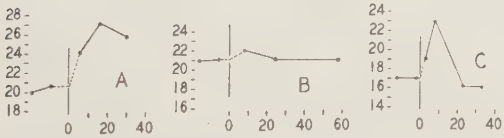

Fig. 1.

The level of adrenalin-like compounds (in gammas per 100 cc) is plotted against time in minutes. The vertical line in A indicates the beginning of a 5-minute electronarcosis, in B the same in a curarized and vagotomized preparation, and in C the beginning of a 2½-minute asphyxiation in a curarized preparation.

In agreement with the observations on patients the level of adrenalin-like compounds rose during electronarcosis (Fig. 1, A). In 9 experiments the mean of the maximal rise in the level was 5 gamma/100 cc. The mean difference between the two control determinations in these 9 experiments was 0.2 gamma/100 cc. Statistical treatment of these data shows that the increase of the level due to electronarcosis is significant at the 5% level ($t = 2.7$).

As shown in Fig. 1, A, the level of adrenalin-like compounds has started to decline again 30 minutes after the end of electronarcosis. It is of interest to note that Kobro[10] observed a fall of the level 30 minutes after an injection of adrenalin.

The initial stage of electronarcosis is characterized by a moderately severe asphyxiation of the animal and by convulsions. Both concomitants can be prevented by the administration of Intocostrin (0.5-0.8 cc/kg) combined with artificial respiration. Furthermore such preparations were doubly vagotomized to prevent the cardiac arrest which usually occurs in the beginning of electronarcosis[9] and which might cause a short systemic asphyxiation. The effect of electronarcosis on the level of adrenalin-like compounds in such preparations was variable, but in general an unusually small rise of the level was found. The results of one of these experiments are given in Fig. 1, B.

Since there was some indication that either convulsive activity or asphyxiation (or both) might cause the increase of adrenlin-like compounds during electronarcosis, the effect of asphyxiation alone was examined. In curarized preparations the trachea was clamped for a total of 2½ minutes (usually with a short interval when the heart action became weak). This caused a rise in the level of the compounds which was of the same order as the increase observed after electronarcosis. Fig. 1, C, shows the results of such an experiment. The mean increase of 4 experiments was 6 gamma/100 cc. In the blood of animals killed by asphyxiation the level rises even more, up to 38 gamma/100 cc has been observed.

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[9] Frostig, J. P., van Harreveld, A., Reznick, S., Tyler, D. B., and Wiersma, C. A. G., *Arch. Neurol.*, 1944, **51**, 232.

[10] Kobro, M., *Acta Med. Scand.*, 1946, **125**, 49.

Discussion and Summary. There is reason to believe that when asphyxiation is prevented electronarcosis causes only small increases of the level of adrenalin-like compounds in blood. It had been found previously[11] when one of two rabbits connected by a double carotid-jugular anastomosis was given an asphyxia-free electronarcosis, that the blood pressure in the other showed a slight increase. This was believed to be due to a release of adrenalin, which in the present experiments may cause the slight rise in the level of "adrenalin-like" compounds during asphyxia-free electronarcosis. It seems likely that the much larger increase of these compounds which is always present in animals subjected to electronarcosis without further precautions, is caused by asphyxiation.

Kobro[12] did not observe any effect of respiratory impairment (letting the animal breathe through a tightly woven cloth) on the adrenalin level. The impairment employed in the present experiments, though of shorter duration, was more severe (clamping of the trachea), which may account for the difference in the results obtained.

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[11] van Harreveld, A., and Dandliker, W. B., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 391.

[12] Kobro, M., *Acta Med. Scand.*, 1946-47, **126**, 97.

16971

Obesity in Albino Mice Due to Single Injections of Goldthioglucose.

GEORGE BRECHER AND SAMUEL H. WAXLER. (Introduced by K. M. Endicott.)

From the Pathology and Pharmacology Laboratory, Experimental Biology and Medicine Institute, National Institutes of Health, Bethesda, Md., and The Army Medical Department Research and Graduate School, Washington, D.C.

During a toxicological study of goldthioglucose, it was noted that a number of albino mice receiving this compound showed a marked increase in size. This response occurred in animals which had been injected with a single dose of this drug. The present investigation was undertaken in an attempt to define the conditions under which these gains in weight occur.

Experimental. Goldthioglucose is an unstable compound of gold and thioglucose, containing 50% gold. It dissociates in the presence of water. The compound is hygroscopic and is therefore utilized as a suspension in sesame oil. The concentration of the suspension used in these experiments was 100 mg of goldthioglucose in 1 cc of the oil (supplied by Schering Corporation, Bloomfield, N.J.). The route of administration was by intraperitoneal or intramuscular injection.

Stock male and female mice were used. The animals were kept on a pellet diet, and fed *ad lib.* In some of these studies the animals were kept in individual cages and the food intake determined. All animals were weighed at weekly intervals.

The LD_{50} of goldthioglucose was 40-50 mg intraperitoneally for mice weighing 20 g. Fifty to 70% of the animals survived doses of 35 mg of goldthioglucose, and 90% survived doses of 25 mg. All animals survived the intraperitoneal injection of 12 mg of the compound. Fatalities from the administration of goldthioglucose generally occurred within the first 3 to 4 days. There was no further mortality due to the toxicity of the drug among those animals which survived one week.

In one representative experiment, animals weighing 18-23 g were given single injections

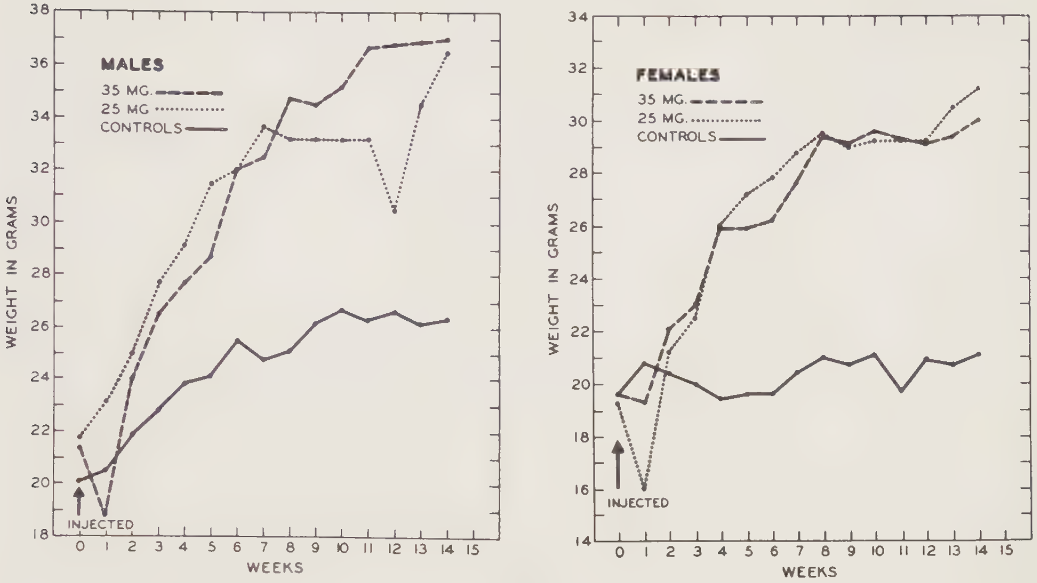

Fig. 1.
Average weights of male and female mice injected with goldthioglucose (25 and 35 mg), compared with controls.

of goldthioglucose intraperitoneally. Thirty males and 30 females were given 35 mg of the compound, and 5 males and 5 females were given 25 mg of the compound. Of the animals injected with 35 mg of goldthioglucose, 15 males and 24 females survived. Of the animals injected with 25 mg of the compound, 4 males and 4 females survived. The average weight of these survivors and of the controls is represented in Fig. 1. The differences in weight gains between treated and untreated animals were statistically significant in both males and females.

In another experiment, some of the control mice were injected with 0.5 cc of sesame oil. This was done to exclude the possibility that the oil of sesame used as the vehicle for goldthioglucose may have an effect on body weight. Out of a group of 25 male mice, 15 were injected intraperitoneally with 25 mg in 0.25 sesame oil. Seven of these survived. Four animals received 0.5 cc sesame oil intraperitoneally, and 5 animals were left untreated. Fig. 2 shows the individual weights of these animals at weekly intervals. The difference in weights of untreated controls and those injected with sesame oil were not statistically significant, while the animals given goldthioglucose in sesame oil showed significantly greater weight gains. An identical experiment with female mice showed similar results.

In order to test the possible influence of the route of administration, one group of mice was given a single dose of 25 mg of goldthioglucose intramuscularly. Because of the small muscle mass available, the dose was split and 12.5 mg of goldthioglucose was given in each thigh. The weight curves of these animals were in all respects similar to those of the intraperitoneally injected animals. Weight increases in the injected group were again significantly higher than in the controls.

In all these experiments only one-third to one-half of the injected animals showed unusual weight gains. This is well shown in Fig. 2, in which the individual weights of animals surviving the injection of 25 mg goldthioglucose are recorded at weekly intervals. In several experiments animals that failed to show significant weight gains by the eighth week were given another 25- or 35-mg dose of goldthioglucose. Significant weight gains were subsequently observed in a number of re-injected animals.

Animals injected with 12 mg of goldthio-

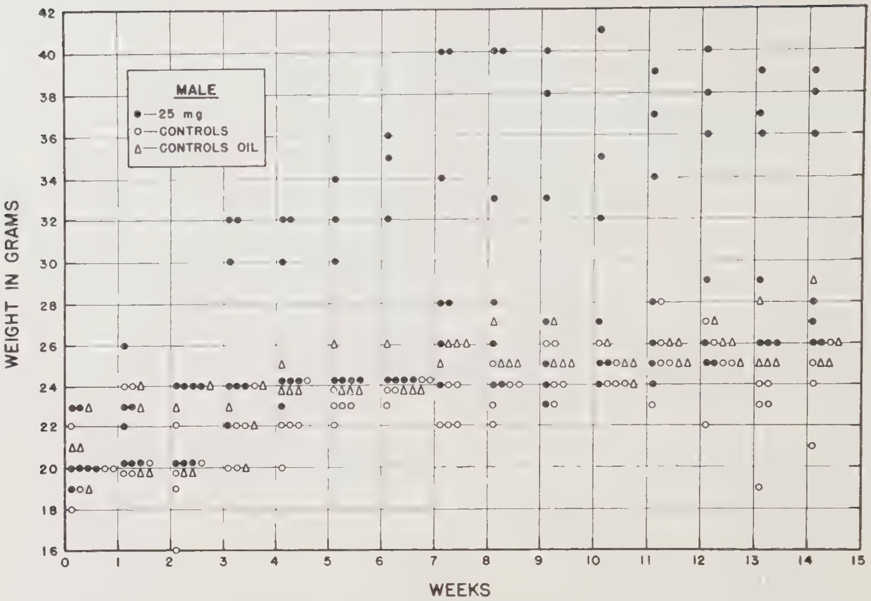

Fig. 2.

Comparison of individual weights of male mice injected with goldthioglucose, sesame oil, and controls.

glucose intraperitoneally showed no significant weight increases as compared with the control animals. Animals given injections of 5 mg of goldthioglucose intraperitoneally twice a week until a total of 150 mg was given, failed to show any significant weight gains.

All animals used in the above experiments were killed at the end of 14 weeks. There was marked increase in intra-abdominal fat and some increase in intrathoracic fat in the heavy animals. There was also a moderate increase in subcutaneous fat in the pelvic region and the region of the shoulder girdle. The viscera and the brain of both injected and control animals were examined microscopically. Mild inflammatory reactions at the site of injection and histochemically demonstrable gold in reticulo-endothial cells of various organs were present in all injected animals. There was mild to moderate centrolobular fatty infiltration of the liver in obese animals. This fatty infiltration ran roughly parallel with the weight of the animals. No other lesions were found in the liver, spleen, pancreas, kidney, adrenal, genital organs, heart, lung, lymph nodes, brain, hypophysis, thyroid, parathyroids, and adipose tissue.

Autopsy findings indicated that the abnormal amount of adipose tissue in injected mice might account for most of their weight gain. To verify this observation, the total body fat content was determined by the gravimetric method, using acid hydrolysis and ether extraction. Nitrogen, water, and ash were determined on aliquots from the same animals. Untreated controls selected at random were used for these determinations and compared with a group of injected animals showing marked weight gains. The weight of the injected animals so selected ranged from 38 to 60 g, and that of the controls from 18 to 25 g. The mean values and standard errors of the analytical determinations in the two groups are given in Table I. These analyses showed that the weight difference between injected heavy animals and non-injected controls could be primarily accounted for by the marked increase in adipose tissue in the injected animals.

Roentgenological examinations of the skeleton of obese and normal animals showed no recognizable difference in size, shape, or density of bones.

Summary. Marked weight gains were produced in stock albino mice by single injec-

TABLE I.
Total Lipids, Protein, Water, and Ash.

	Normal mice (7 animals)					Injected mice showing marked wt gains (7 animals)				
	Wt, g	Lipids g	Protein g	Water g	Ash g	Wt, g	Lipids g	Protein g	Water g	Ash g
Mean	22.1	2.42	3.65	16.2	.74	45.6	18.07	5.55	20.0	.72
Stand. error	.9	.33	.12	.7	.05	2.4	1.94	.26	.9	.09

tions of goldthioglucose. Analysis of total body lipids, protein, water, and ash showed these weight gains to be primarily due to an increase in adipose tissue. This obesity occurred in approximately one-third of the survivors of sublethal doses of goldthioglucose. Except for centrolubular fatty infiltration of the liver in obese animals, no anatomic lesions were found in the organs examined.

16972

Effect of Aminophylline on Plasma Prothrombin and Ac-Globulin in Dogs.

HARRY M. MCCORMICK AND IRVING I. YOUNG. (Introduced by Walter H. Seegers.)*
From the Department of Physiology and Pharmacology, Wayne University College of Medicine, Detroit, Mich.

Several conflicting reports have appeared in recent literature concerning the effect of the administration of methylxanthines on prothrombin activity. Field and coworkers[1] reported a hyperprothrombinemia following oral administration of these drugs to experimental animals in doses ranging from 10-400 mg/kg body wt. Scherf and Schlachman[2] also reported a shortening of prothrombin time in human subjects given therapeutic doses of aminophylline and theophylline with sodium acetate either orally or intravenously. However, a number of investigators have been unable to confirm these results. Quick[3] noted no increase in the prothrombin level of dogs and rabbits fed large doses (100-200 mg/kg) of methylxanthines. In a careful study, Holland and Gross[4] reported that methylxanthines, when given to dogs either in single intravenous doses of 25-50 mg/kg, or in daily oral doses of 20 mg/kg, produced no shortening in prothrombin time. Furthermore, single intravenous doses of 0.5 g of aminophylline failed to shorten the prothrombin time of human subjects. Several other studies[5-8] with human subjects failed to demonstrate significant changes in prothrombin time after oral or intravenous administration of aminophylline. All studies reviewed above were done by using only one-stage methods for the determination of prothrombin. Either Quick's original technique or one of its numerous modifications were utilized.

Because of recent advances in the under-standing of the blood clotting mechanism, and improved methods of analysis,[9–13] the problem was investigated further in this laboratory. It seemed especially important to take into account the newly recognized factor which accelerates the conversion of prothrombin to thrombin. This accelerator (Ac-globulin) is now recognized to be an integral part of the coagulation mechanism. A quantitative method for measuring its concentration has been developed.[12] It is essentially an adaptation of the two-stage method for prothrombin determination. The original two-stage method for prothrombin determination has also been modified[13] to account for variation in the activity of Ac-globulin. Using these newer techniques, we have, in this study, been able to demonstrate a definite effect of aminophylline on the coagulation mechanism in dogs.

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* Aided by a grant from the United States Public Health Service.

[1] Field, J. B., Larsen, E. G., Spero, L., and Link, K. P., *J. Biol. Chem.*, 1944, **156**, 725.

[2] Scherf, D., and Schlachman, M., *Am. J. Med. Sc.*, 1946, **212**, 83.

[3] Quick, A. J., *J. Biol. Chem.*, 1945, **161**, 33.

[4] Holland, M. S., and Gross, E. G., *J. Iowa St. Med. Soc.*, 1948, **38**, 183.

[5] Breytspraak, R. W., and Greenspan, F. S., *Am. J. Med. Sc.*, 1946, **212**, 476.

[6] Poindexter, C. A., and Meyers, L., *Quart. Bull. Northwestern Univ. Med. School*, 1946, **20**, 130.

[7] Gilbert, N. C., Dey, F., and Trump, R., *J. Lab. and Clin. Med.*, 1947, **32**, 28.

[8] Blood, D. W., and Patterson, M. C., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 130.

Methods. Following a control period of 2 to 4 days, aminophylline was administered intravenously in varying dosages to healthy dogs maintained on a stock diet. Blood samples were drawn 4, 24, 48, and 72 hours after administration of the drug, and subsequently at 2 to 3 day intervals until normal values were obtained. To avoid thrombin formation we observed ordinary precautions in withdrawing blood and followed largely the technique described elsewhere.[14] One part of 3.2% sodium citrate was added to 9 parts blood. The plasma was removed following centrifugation and stored in a frozen state at —20°C until determinations were performed. Serial hematocrit determinations were made at first but because no significant variations were noted, this was later discontinued. The clotting time of whole blood, using the Lee-White method, was also followed at frequent intervals.

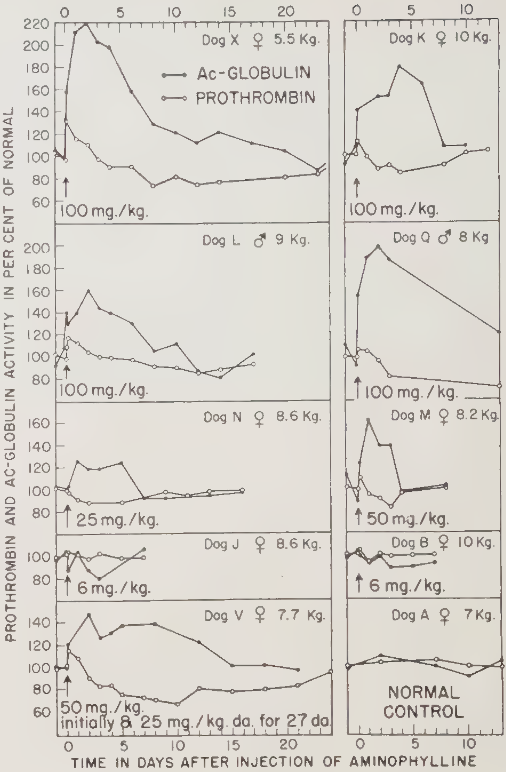

Fig. 1.

The protocol shows changes in plasma Ac-globulin and prothrombin concentration for 9 experimental animals and one typical control.

Prothrombin activity was determined by the modified two-stage method described by Seegers,[13] and Ac-globulin activity was estimated by the method of Ware and Seegers.[12] All determinations were compared to a standard control run simultaneously, so that minor corrections could be made for variations in reagents.

Results. Fig. 1 illustrates the results obtained with 8 dogs given single, intravenous doses of aminophylline, and with one dog given multiple intravenous injections. Prothrombin and Ac-globulin activities are expressed in per cent of normal control values. Expressed in units per cc of citrated plasma, normal prothrombin activity ranged from 170 to 185; Ac-globulin activity from 185 to 230.

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[9] Fantl, P., and Nance, M., *Nature*, 1946, **158**, 708.

[10] Owren, P. A., The Coagulation of Blood, Investigations on a New Clotting Factor, Oslo, 1947.

[11] Ware, A. G., Guest, M. M., and Seegers, W. H., *J. Biol. Chem.*, 1947, **231**, 169.

[12] Ware, A. G., and Seegers, W. H., *J. Biol. Chem.*, 1948, **172**, 699.

[13] Seegers, W. H., Blood Clotting and Allied Problems, Josiah Macy, Jr. Foundation, Feb., 1948.

[14] Fahey, J., Olwin, J. H., and Ware, A. G., Proc. Soc. Exp. Biol. and Med., 1948, **69**, 491.

All animals were first observed for a control period of a week or so to establish their particular normal (100%) levels.

Following the administration of 100 mg of aminophylline per kg body wt, a small but consistent rise in prothrombin activity was noted in 4 hours, falling to normal by 24 to 48 hours (dogs X, Q, L, and K). This was followed by a drop to approximately 15 to 30% below normal, gradually returning to normal in about 2 to 3 weeks. Ac-globulin activity rose sharply in 4 hours, reached a maximum in 48 to 96 hours, and then gradually fell to normal levels. Determinations on blood drawn 2 hours after administration of aminophylline to dog L showed that a significant response was present even at this time. This characteristic pattern was less evident with smaller doses (50 and 25 mg/kg body wt). Administration of 6 mg/kg body wt (approximate therapeutic dosage in man) was without effect on either prothrombin or Ac-globulin activity (dogs B and J).

Several dogs given salts of caffeine and theobromine intravenously in large single doses (100 mg/kg body wt), were found to respond in the same characteristic manner.

Dog V, given 50 mg of aminophylline per kg body wt initially, and then daily doses of 25 mg/kg body wt for 4 weeks, demonstrated that the elevation in prothrombin activity could not be maintained by repeated injections. Increased Ac-globulin activity persisted for a longer period but returned to normal in spite of continued administration of the drug.

As a matter of interest, plasma from several of these dogs was analyzed for prothrombin activity using the one-stage method. The technique originally described was carefully followed, using 100% plasma. Deviations from the normal values of 6 to 6.5 seconds were found with the large intravenous doses of the drug. This paralleled the results obtained using the two-stage method. For example, the prothrombin time of dog L fell to 5 seconds 4 hours after administration of aminophylline, remained at 5½ seconds for 96 hours, and increased to 7 seconds before returning to normal. On the other hand, dog J showed no deviation from normal.

No correlation could be found between the Lee-White clotting time of whole blood and variations in prothrombin and Ac-globulin activities. This has also been noted by other authors and is undoubtedly due to inadequacies of the method. A modified technique has been described by Blood and Patterson[8] which is probably a considerable improvement over the method we used. These investigators consistenly obtained a very narrow range of normal values for the clotting time of whole blood.

Summary. 1. Large doses of aminophylline, administered intravenously to dogs, produced a transient elevation in prothrombin activity, followed by a late hypoprothrombinemia with return to normal in about 2 to 3 weeks. Simultaneously, a more marked and persistent rise in Ac-globulin activity occurred. Small doses of 6 mg/kg body wt were without significant effect.

2. No correlation could be found between the Lee-White whole blood clotting time and variations in prothrombin and Ac-globulin activity.

16973

Effects of Adrenoxyl on Blood Coagulation Mechanism and Vasomotor Response.

Edwin J. Pulaski, Hans Reichel, and Arthur B. Voorhees, Jr.
(Introduced by J. A. Shannon.)
From the Surgical Research Unit, Brooke General Hospital, Brooke Army Medical Center, Fort Sam Houston, Texas.

Cannon and Mendenhall[1] have shown that adrenalin shortens the coagulation time of blood. Roskam, Derouaux and associates[2] studied the effect of small doses of adrenalin on the bleeding time and reported a hemostatic action following the administration of the drug.

Adrenoxyl is a relatively stable derivative of adrenalin* developed by Braconier and associates.[3] Adrenochrome, adrenoxime,[4,5] and adrenoxyl,[6] oxidative derivatives of adrenalin, are reported as being capable of reducing the bleeding time approximately 25%. The adrenoxyl effect is demonstrable 15 minutes after intravenous injection in rabbits and 30 minutes after injection in man. The optimal doses are 0.001 mg in the rabbit and 0.1 to 0.5 mg in man. Derouaux[6] and Bacq[7] report that the circulation in rats and rabbits remains unaltered by the above doses of Adrenoxyl. These investigators emphasize lack of any sympathomimetic effect as reflected by the absence of change in the blood pressure and pulse rate in animals following injection of the drug. Roskam and associates believe that Adrenoxyl has a vasomotor effect which is responsible for the rapid occlusion of injured vessels. On the other hand, Desfontaines[8] has found a vasodilator effect of adrenochrome in man and recommends it for the treatment of hypertension.

As a preliminary study of the pharmacological properties of Adrenoxyl in relation to the bleeding time, we followed the administration of the drug with these screening procedures: Clotting time; prothrombin time; pulse wave velocity; skin temperature; and bleeding time determination. Such procedures would yield information concerning alterations in coagulability of blood and the vasomotor response of the injured vessels.

Methods and Results. Adrenoxyl prepared by Labaz Laboratories in ampules containing 0.1 and 0.5 mg in solution, was used in this investigation.† The prothrombin time and bleeding time determinations were performed on rabbits of both sexes. All other studies were made on healthy men between 17 and 39 years of age.

1. *Clotting time* (Table I). Blood samples were taken from the arm veins of 6 seated individuals and drawn directly into capillary tubes by a method devised by Pichotka and Reichel.[9] The method consists of drawing blood from a vein by a 22 gauge needle connected to a capillary tube by a paraffin joint. The method is designed to limit the possible errors caused by surface and tissue factors. The capillaries were broken every 30 seconds. Three to 5 determinations were considered to be sufficient to give mean values under conditions of

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* The monosemicarbazone of adrenochrome.

[1] Cannon, W. B., and Mendenhall, W. L., *Am. J. Physiol.*, 1914, **34**, 225.

[2] Derouaux, G., and Roskam, J. Physiol. Soc., June 5, 1937, through *J. Physiol.*, 90: Proc., 65, 1937.

[3] Braconier, F., LeBihan, H., and Beaudet, C., *Arch. Internat. de pharmacodyn. et therap.*, 1943, **69**, 181.

[4] Green, D. E., and Richter, D., *Biochem. J.*, 1937, **31**, 597.

[5] Derouaux, G., *Arch. Internat. de pharmacodyn. et therap.*, 1941, **66**, 202.

[6] Derouaux, G., *Arch. Internat. de pharmacodyn. et therap.*, 1943, **69**, 142.

[7] Bacq, Z. M., *Presse med.*, 1947, **16**, 175.

[8] Desfontaines, G., *Ann. biol. clin. Par.*, 1948, **6**, 138.

[9] Pichotka, J., and Reichel, H., unpublished data.

† Supplied by Squibb Institute for Medical Research, New Brunswick, N. J.

TABLE I.
Effect of Adrenoxyl on Clotting Time.

	Control							
Case	No. of determinations	Avg value (sec.)	Possible deviation (sec.)	Dose (mg)	Time after injection (min.)	No .of determinations	Avg value (sec.)	Possible deviation (sec.)
1	3	330	±30	.10	10-50	5	362	±52
2	3	470	±60	.45	10-45	4	420	±30
3	4	405	±45	.50	15-20	4	435	±75
4	3	370	±30	.45	15-20	4	367	±37
5	5	450	±60	.25	10-40	4	510	±45
Avg		405	±45				419	±45

TABLE II.
Effect of Adrenoxyl on Prothrombin Time.

Rabbit No.	Normal Prothrombin time (sec)	Dose	Post Adrenoxyl time (min.)	Prothrombin time (sec.)	Change
2	18 18 18 19 19	1	20	18 19	0
4	19 17 19 18 19	1.5	40	20 24 23 20	Increase, slight
8	17 17 17 17 16	3	45	clotted	– – – –
4	18 16 18 17	3	30	19 17 17 18 16	0
8	19 18 19 18 19	1	30	19 17 19 19 19	0
5	19 18	0.5	30	19 19	0

study. After the control measurements Adrenoxyl in a dosage of 0.10 to 0.50 mg was injected intravenously, and the clotting time of four to five separate blood samples was measured at intervals varying between 10 to 50 minutes later.

The clotting time had an average standard deviation of 4.4% in each subject in the control, and a mean standard deviation from the corresponding normal values of 4.8%. The individual differences between the pre- and post-Adrenoxyl findings fall within the range of physiological deviation.

2. *Prothrombin time* (Table II). Determinations were made on 5 rabbits before and after Adrenoxyl administration on blood samples obtained by intracardiac tap at stated intervals. The samples were collected in dry syringes and transferred immediately into test tubes containing 0.1 molar sodium oxalate. Prothrombin time was determined by the Quick method.[10] The applied dosage of Adrenoxyl varied between 0.0005 and 0.003 mg. No essential change was noted in the prothrombin time following Adrenoxyl injection.

3. *Pulse wave velocity* (Table III). Determinations were carried out by the photographic method of Frank[11] and Wexler.[12] Rubber membrane capsules and rubber tubes were used for air transmission of the impulses. The mirrors on the membranes were illuminated by a light beam from a projector, provided with a 3-slit lantern slide. The pulse curves of the carotid, femoral, and radial arteries were recorded by means of a photo-electric highspeed kymograph. The pulse wave velocity of the aorta (CA) and of the brachial (CB) artery was computed from the difference between the rise of the carotid, the femoral, and the radial curves. From these values the quotient, CB/CA, was calculated. Pulse rate and the blood pressure were also measured. Control determinations were followed by intravenous injections of 0.15 to 0.50 mg Adrenoxyl. The pulse rec-

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[10] Quick, A. J., *Amer. Journal Clin. Path.*, 1940, **10**, 222.

[11] Frank, O., *Z. f. Biol.*, 1903, **44**, 445; 1905, **46**, 441; 1929, **89**, 289.

[12] Wezler, K., *Z. f. Biol.*, 1935, **96**, 261; *Z. f. Breislaufforsch.*, 1935, **27**, 721.

TABLE III.
Effect of Adrenoxyl on Circulation.

Case	Dose	Time of determinations (min after inj.)	PR—Beats per minute	BP (mm Hg)	CA (m/sec.)*	CB (m/sec.)*	CB/CA
6	.15	Resting	74	120/70	6.37	7.45	1.17
		20	80	125/70	6.75	8.10	1.20
7	.20	Resting	60	100/65	4.05	4.95	1.22
		10	60	115/70	4.12	5.03	1.22
8	.20	Resting	81	120/75	5.55	6.42	1.16
		10	70	120/70	5.18	5.77	1.11
9	.10	Resting	69	110/70	5.90	7.97	1.35
		60	67	110/70	5.74	7.80	1.36
10	.10	Resting	51	115/70	6.27	6.41	1.02
		50	55	115/70	6.25	6.49	1.04
11	.50	Resting	52	110/70	6.63	9.54	1.44
		15	54	110/70	6.26	9.14	1.46
12	.50	Resting	45	110/70	5.50	7.62	1.38
		15	43	115/70	5.78	7.68	1.33
13	.50	Resting	66	120/75	6.80	9.04	1.31
		15	67	120/75	7.04	9.12	1.30

* Meters per second.
PR—Pulse rate.
BP—Blood pressure.
CA—Pulse wave velocity in aorta.
CB—Pulse wave velocity in brachial artery.

TABLE IV.
Effect of Adrenoxyl on Skin Temperature.

Case	Dose (mg)	Time after injection (min.)	Mean skin temp. (°C) Normal	Mean skin temp. (°C) Adrenoxyl	Room temp. (°C)
12	.5	40	31.85	31.44	28.7
14	.5	25	31.11	31.01	26.9
15	.5	30	31.60	31.73	27.7
16	.5	40	30.80	30.70	27.9

ords were repeated immediately and 10-50 minutes later. During the whole procedure the subjects were maintained in the supine position. Following the injection of Adrenoxyl, deviations of the pulse wave velocity were negligible. Maximal change of the quotient, CB/CA, did not exceed 35%, which falls within physiological variations.

4. *Skin temperatures* (Table IV). Readings were taken from undressed recumbent subjects by means of a Dermalor instrument having a standard deviation of ± 0.2° C. The control readings were performed 30, 40, and 50 minutes after the undressing, in a room temperature of 27° to 29° C. The skin temperature measurements were repeated 20, 30, and 40 minutes after an intravenous injection of 0.5 mg Adrenoxyl. The mean value was determined for each selected body site (forehead, ears, nose, chin, shoulders, forearm, hand, fingers, umbilicus, knee, mid-leg, feet, and toes) on the basis of 3 "control" and 3 "test" measurements. Skin temperature determinations remained essentially unchanged following administration of Adrenoxyl.

5. *Bleeding Time.* Determinations were made on 12 rabbits anesthetized by sodium amytal, intravenously administered. Small, uniform, through and through incisions were made along the caudal margin and tip of the ears. The incised wounds were placed under a uniform saline drip. The time was recorded between the moment of incision and the moment of cessation of bleeding. The error of cutting a large vessel was avoided by transillumination. After a satisfactory control time was established, Adrenoxyl (0.001 to 0.0025 mg) was injected intravenously and periodic bleeding time determinations were

made over the ensuing 60 minute period. No significant reduction in bleeding time was noted following the injection of Adrenoxyl.

Discussion. Insofar as venous clotting time and prothrombin time reflect the speed of fibrin production, Adrenoxyl does not foster an accelerated process under the stated experimental conditions. It is, however, conceivable that tissue thromboplastic activity can be altered by Adrenoxyl and that the alteration is not evident where venous or heart sampling reduces the amount of tissue thromboplastin to the minimum.

After administration of Adrenoxyl, observations of the pulse rate, the blood pressure, the arterial wall tonus (as reflected by the pulse wave velocity), and the vaso-motor response of the superficial vessels (as evaluated by skin temperature determinations), do not reveal data which would suggest alteration in the vasomotor response that would not fall within normal physiological limits.

Bleeding time experiments are inconclusive since the experimental error of the technique used is of such great magnitude. We were unable to isolate real evidence that Adrenoxyl reduces the bleeding time under the stated experimental conditions. It is interesting to us to note that when an animal under light anesthesia responds to the stimulus of an incision on the ear, the bleeding time is reduced. This finding raises the question of advisability of collecting bleeding time data on unanesthetized animals.

Conclusions. 1. Under the stated experimental conditions Adrenoxyl does not influence (a) the speed of the coagulation process, and, (b) the vasomotor response.

2. The method of bleeding time determination in the experimental animal in our hands does not give conclusive results, therefore we are unable to confirm or deny the findings of Roskam and associates.

16974

Evaluation of Certain Dangers in the Use of Jet Injection Technic.

ROBERT V. BROWN. (Introduced by J. P. Quigley.)
From the Division of Pharmacology, University of Tennessee, Memphis.

As a substitute for the hypodermic syringe, a jet injector which utilizes the principles of the Diesel fuel injector has been described by Hingson and Hughes.[1] In the administration of substances by the hypodermic syringe, the possibility of an accidental intravenous injection is recognized. The present study was designed to investigate the possibility of a similar accidental intravenous injection with the "Hypospray."

Experimental Work. Dogs anesthetized with 30 mg per kg of sodium pentobarbital intravenously were given atropine sulfate by vein until electrical stimulation of the cut right vagus nerve failed to slow the heart. Both jugular veins were exposed by a medial approach so they could be seen by eversion of the edges of the incision. Blood pressure was continuously recorded by a mercury manometer connected to one carotid artery. Known doses of epinephrine were injected by syringe into the right jugular vein.

A "Hypospray"* with a 125 pound spring was loaded with a cartridge† containing 0.25 cc of a 0.4% solution of epinephrine hydrochloride. By eversion of the edge of the incision the left jugular vein was exposed to view. The tip of the cartridge was then pressed firmly against the skin on outside of the shaved neck and so aligned that the vein lay directly between the cartridge tip and the

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[1] Hingson, R. A., and Hughes, J. G., *Anesth. and Analg.*, 1947, **26**, 221.

* Courtesy of the R. P. Scherer Corp., Detroit.

† Courtesy of the Gelatin Products Corp., Detroit.

TABLE I.
Summary of Jet Injections Lodging as Much as One Per Cent Intravenously.

		Blood pressure (mm Hg)		μg epinephrine base lodged in vein			
Dog No.	Wt	Control	Maximum	Per kg	Per dog	%*	State of vein
1	5.5	96	226	18.74	103	15.2	D†
2	15.9	126	220	.49	8	0.9	D
		144	315	51.48	818	97.7	Empty
		135	306	35.32	562	66.8	D
3	5.0	128	247	10.34	52	6.2–	Empty
		122	240	8.09	40	4.8	D
4	4.7	108	188	3.38	16	1.9	D
		110	195	3.48	16	2.0	D
5	16.0	153	206	.52	8	1.0–	D

* Per cent of the total epinephrine available, *i.e.*, 833 μg.
† Distended.

incision. Any of the jet passing through the vein would escape into the room or be sponged out at once. The instrument was then discharged. The vein rested directly on the subcutaneous tissue or else was separated from it by about 5 mm of muscle. For some jet injections the jugular vein was emptied; for others the vein was distended by clamping and the clamps were removed immediately after the discharge.

A dose-effect curve was fitted for each animal by the method of least squares, using the equation: maximum blood pressure attained is equal to a constant multiplied by the dose per kilogram raised to a constant power. Accurate assays of epinephrine can be made from such dose-effect curves.[2–8] The maximum blood pressure reached after each jet discharge of epinephrine was measured. From the dose-effect curve or its equation the quantity of epinephrine required to produce the given maximum pressure can be determined either graphically or by calculation. The results of 12 jet injections which lodged intravenously as much as 1% of the epinephrine in the cartridge are listed in Table I; 22 discharges which lodged less than 1% are omitted.

Results and Discussion. The greatest amounts of the drug which lodged in the vein in 34 jet injections were 97.7, 66.8, 15.2, and 6.2% of the total discharged. Of these, the first and fourth quantities lodged in empty veins; the second and third lodged in distended veins. Thus if the jet is discharged over a large vein a significant fraction of the discharged drug may be administered intravenously.

When the "Hypospray" is used, several factors appear to determine the fraction of the epinephrine which lodges in the veins: (1) alignment of the blood vessel with the jet, (2) amount of pressure applied to the tissues, (3) diameter of the vein, (4) amount and toughness of the tissues separating the nozzle and the vein lumen, (5) ability of the vein to roll away from the discharge under impact of the jet.

Even though the vein was visible, we were not always successful in discharging the jet through the diameter of the vein. When the jet nicked the vein edge, little of the drug entered the lumen as the major portion was deflected. Further, such off-center discharges tended to roll the vein to one side.

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[2] Elliott, T. R., *J. Physiol.*, 1904-5, **32**, 401.

[3] *Ibid.*, 1912, **44**, 374.

[4] Lyon, D. M., *J. Pharmacol. and Exp. Therap.*, 1923, **21**, 229.

[5] Rosenblueth, A., *Am. J. Physiol.*, 1932, **101**, 149.

[6] Clark, A. J., Mode of Action of Drugs on Cells, Baltimore, 1933.

[7] Hjort, A. M., de Beer, E. J., and Randall, L. O., *J. Pharm. and Exp. Therap.*, 1941, **71**, 105.

[8] Brown, R. V., *Fed. Proc.*, 1948, **7**, 208.

Conversely, with direct hits the vein was seen to balloon out whether it was originally full or empty. Firm pressure of the cartridge tip against the tissues did not prevent such ballooning. After having passed through the proximal vein wall, the jet had lost so much velocity that much of it failed to penetrate the distal vein wall and thus remained in the lumen.

Inspection of Table I shows that the amount of the drug retained was not appreciably influenced by whether or not the vein was empty or full. In larger animals the veins are larger and are easier to hit through the diameter. Also the thicker vein walls decreased the jet velocity and less escaped through the distal wall. Since man has numerous large superficial veins, his risk of an intravenous injection may be greater than that of the dog when the jet is used.

The portion of a vein penetrated by a jet of epinephrine became blanched and constricted for several minutes. Sometimes small clots appeared on the vessel at the points of entrance and exit of the jet.

The shape of the blood pressure curve resulting from a jet injection of epinephrine differed from that following needle injection. With direct hits, the ascending limbs of the curves were similar, but the blood pressure always returned to the control level more slowly after a jet injection. This is taken to indicate: (1) a gradual entrance into the lumen of epinephrine lodged in the wall of the vein, (2) entrance of additional small quantities lodged in the small tributaries, (3) rapid absorption of epinephrine trapped in adjacent tissues (*cf.* Clark.[9]).

Massage of a jet injected muscle, connective tissue or even the vein itself elevated the blood pressure. In one case, the blood pressure rise induced by massage of a jet injected area was 12 mm of Hg, but lower responses were usually obtained. Luckhardt and Koppanyi[10] have reported a similar elevation of blood pressure from massage of areas containing needle-injected epinephrine.

As the blood pressure slowly returned to the control level, cardiac irregularities, showing as dropped beats, were much more frequent from jet injections than from needle injections. It is not known whether the dropped beats indicated premature systoles of the ventricles or heart block.

Ten attempts to make blind intravenous injections with the "Hypospray" at the junction of the jugular and subclavian veins or into the femoral vein were not successful.

Conclusions. 1. Under conditions very favorable for intravenous injection, the "Hypospray" has lodged as much as 97% of the total available drug in the vein, even when administered through the skin and subcutaneous tissues.

2. Large veins are easier to hit than small veins and retain a larger amount of the jet-injected material.

3. The probability of unintentionally penetrating a major vessel with the jet is small but the risk is always present.

4. Jet discharges should not be made in the neighborhood of large vessels unless the drug to be injected is safe for intravenous administration.

5. The jet may produce macroscopically visible damage to a vessel.

The author wishes to thank Drs. J. P. Quigley and R. A. Woodbury for their interest and help.

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[9] Clark, A. J., and Raventós, J., *Quart. J. Exp. Physiol.*, 1939, **29**, 185.

[10] Luckhardt, A. B., and Koppanyi, T., PROC. SOC. EXP. BIOL. AND MED., 1926, **23**, 774.

16975

Mammary Cancer in Castrate Male Mice Receiving Ovarian and Hypophyseal Grafts at Various Ages.*

Ruth Silberberg and Martin Silberberg.

From the Snodgras Laboratory of Pathology, St. Louis City Hospital, St. Louis, Mo.

In young virgin mice of susceptible strains, grafts of anterior hypophysis stimulate the growth of the mammary gland and increase the incidence of breast cancer.[1] Anterior hypophyses grafted together with ovaries are ineffective in young males with intact testicles; however, these transplants induce mammary cancer in young castrate males. This carcinogenic effect has been attributed to the gonadotropin given off by the transplanted hypophyses and stimulating the output of estrogenic hormone by the animal's own or by the grafted ovaries.[1,2] This view is based on the fact that in the absence of ovaries hypophyseal grafts are ineffective. The reaction of the breast tissue of male castrates to transplanted ovaries alone is definite, and the number of mammary tumors appearing in these animals may even exceed the number of spontaneous breast cancers found in virgin females of the same strain.[2-5] Moreover, the response of the male breast to injections of estrogenic hormone depends upon the age of the animal at the beginning of the treatment. A maximum susceptibility was noted if the treatment was started about the onset of sexual maturity. Loeb[6] attributed this age effect to the more marked growth reactivity of the breast of younger animals and possibly—since in females such an age factor could not be demonstrated—to an inhibiting action exerted by the male sex hormone. We found this age effect to be due only partly to the action of the testicle, since in castrates of various ages it was also present, though to a lesser degree.[7]

The present experiments were carried out in order to determine whether or not an age factor operates also in the production of breast cancer in male castrates bearing grafts of ovaries alone or in combination with anterior hypophyses.

Material and Methods. One hundred sixty-four male mice of the closely inbred strain A raised in our laboratory and originating in the Bar Harbor colony were used. The animals were castrated at 3 to 4 weeks of age and received the following grafts at the age of 1 or 7 months respectively: (a) 4 anterior hypophyses (27 young and 28 adult animals); (b) 2 or 4 ovaries (28 young and 26 adult animals); (c) 4 anterior hypophyses and 2 or 4 ovaries (26 young and 29 adult animals). The organs to be grafted were usually obtained from mice 2 to 3 months old and closely related to the host, that is, from brothers, sisters, and cousins (syngenesiotransplants)[1]; in 38 instances, non-related donors (homoiotransplants) were used. At the time of transplantation, the hypophyses and ovaries were removed from the donor animals under sterile precautions and kept in sterilized Ringer's solution for a few minutes until two subcutaneous pockets had been prepared on either side of the chest wall of the hosts. The grafts were then transferred into these pockets, half of them to the right and the other half to the left side.

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* This investigation was supported by a research grant from the National Cancer Institute of the National Institute of Health, U. S. Public Health Service.

[1] Loeb, L., and Kirtz, M. M., *Am. J. Cancer*, 1939, **36**, 56.

[2] Loeb, L., Blumenthal, H. T., and Kirtz, M. M., *Science*, 1944, **99**, 230.

[3] Murray, W. S., *J. Canc. Res.*, 1928, **12**, 18.

[4] de Jongh, S. E., and Korteweg, R., *Acta brev. Neerl.*, 1935, **5**, 12.

[5] Huseby, R. A., and Bittner, J. J., Proc. Soc. Exp. Biol. and Med., 1948, **69**, 32.

[6] Loeb, L., *Biol. Sympos.*, 1945, **11**, 197.

[7] Silberberg, M., and Silberberg, R., (a) Proc. Soc. Exp. Biol. and Med., 1948, **69**, 438; (b) *Arch. Pathol.*, in press.

TABLE I.

Incidence of Breast Tumors and the Time of Appearance of the Neoplasms in the Various Experimental Groups.

Group	Castrates bearing	Age at transplantation	No. of mice	Age at death of all mice (mo.)		Animals with breast cancer			
				Mean	Range	No. of cases	%	Age (mo.) of appearance	
								Mean	Range
I.	Hypophyseal transplants	1 mo.	28	16.1	8-21	0	0		
II.	Ovarian transplants	1 "	27*	16.9	6½-22	1	5	6.5	
III.	Hypophyseal and ovarian transplants	1 "	26	17.1	8-22	8	32.5	12.9	8-20
IV.	Hypophyseal transplants	7 "	28	19.1	8-25	0	0	—	—
V.	Ovarian transplants	7 "	26†	15.9	7½-20	0	0	—	—
VI.	Hypophyseal and ovarian transplants	7 "	29	15.8	9-24	0	0	—	—

*7 animals still alive without tumor } 21-24 mo. old
†4 " " " " "

The wounds were subsequently closed with silk sutures and the animals caged in groups of 4.

The mice were fed a stock diet of Purina Laboratory Chow and water available at all times; they were examined at regular intervals for the appearance of breast tumors or evidence of leukemia. Animals that had developed tumors or were suspected of having leukemia or any other disease were killed. At necropsy, special care was taken to recover the transplants, and definite grafts, as well as tissues suggestive of being remnants of grafted tissue were preserved for microscopic examination. In addition, mammary glands, pieces of liver, spleen, kidneys, lung, heart, thyroid, adrenals, hypophysis, prostate and some bones were removed as well as any pathological structures that were found. The histological findings and the data on leukemia will be reported elsewhere.

Observations. The results of our experiments are presented in Table I.

Age group A. Animals castrated at the age of 3 to 4 weeks and receiving grafts at the age of one month:

I. *Castrates bearing hypophyseal transplants* (28 animals) died or were killed at a mean age of 16.1 months with a range from 8 to 21 months. None of these animals showed a breast cancer. II. *Castrates bearing ovarian transplants* (27 animals) reached a mean age of 16.9 months and a range from 6½ to 22 months. One of these animals (5%) developed a breast cancer at 6½ months of age. At the time of this report 7 animals of this group, 22 to 24 months old, are still alive and without tumor. III. *Castrates bearing ovarian and hypophyseal transplants* (26 animals): The mean age at death was 17.1 months with a range from 8 to 22 months. In 8 mice (32.5%), breast cancers were found. The mean age at death of tumor-bearing animals was 12.9 months, the earliest tumor appearing at 8, the latest at 20 months of age.

Age Group B. Animals castrated at the age of 3 to 4 weeks and receiving grafts at the age of 7 months:

IV. *Castrates bearing hypophyseal transplants* (28 animals) reached a mean age of 19.1 months with a range from 8 to 25 months. None of these mice had breast cancer. V. *Castrates bearing ovarian transplants* (26 animals) did not do well, the mean age at death being 15.9 months with a range from 7½ to 20 months. Again, no breast cancer appeared in any of the animals. Four mice of this group 21 months old and older are still alive and without tumor at the time of writing. VI. *Castrates bearing ovarian and hypophyseal transplants* (29 animals) reached a mean age of 15.8 months with a range from 9 to 24 months. None of these mice had a mammary cancer.

Tumor development was independent of the number of ovaries grafted inasmuch as tumors were found in animals that had received 2 or 4 ovaries in addition to the hypophyseal transplants. The presence or absence of tumor could not be correlated to the state of preservation of the grafted tissue at the time of necropsy. In a number of animals without breast cancer functioning transplants were found at the time of necropsy, whereas in some animals with breast cancer no remnants of transplanted tissue could be detected. The degree of relationship of host and donor apparently did not influence the development of tumors, since breast cancer was found in animals bearing grafts from unrelated donors. This is probably due to the fact that the strain had been closely inbred for years and that the individuals have reached a state of close genetic similarity.

Discussion. The incidence of spontaneous breast cancers in breeding females of our strain A stock was 49%. The earliest breast cancer appeared at 6½ and the latest at 20 months of age with a mean age of 11.4 months. No spontaneous breast cancers have developed in our virgin females of strain A.

No breast cancer was observed in castrate male mice of strain A bearing transplants of anterior hypophyses. This indicates that no direct stimulation was exerted on the mammary tissue by hypophyseal activity or that any existing stimulus[8,9] was too weak to induce cancerous growth.

Of 20 male castrates bearing ovarian grafts one developed a breast cancer. This incidence is considerably lower than that observed by Loeb[2] and by Huseby and Bittner,[5] but approaches more closely the results of Murray.[3] The comparatively small number of carcinomas observed in our experiments is also in agreement with the failure of our virgin female mice of this strain to develop spontaneous mammary cancer. Moreover, castrate male mice showed a maximum susceptibility to estrogen-induced cancer if the injections were begun about the age at which sexual maturity is reached in animals with intact testicles.[7] Similar conditions may exist in regard to estrogenic hormone secreted by the transplanted ovaries. The mice used in the present investigation might not have reached the age of maximum susceptibility at the time the transplants began to function. The susceptibility of the breast tissue at the onset of stimulation seems to play a more important role even than the length of time during which the stimulus is allowed to act. The only tumor found in castrate males bearing ovarian transplants occurred at the age of 6½ months, that is 5½ months after grafting and as early as the earliest cancer in our breeding females. Apparently, the breast tissue of this animal was, for reasons unknown, so highly sensitized that a comparatively slight stimulus of relatively short duration sufficed to produce cancerous growth. In castrate male mice bearing hypophyseal transplants in addition to ovarian grafts the breast cancer rate was 32.5%, that is, about 6½ times as high as in castrates bearing ovarian transplants only. This indicates a marked stimulation of ovarian activity by the grafted anterior hypophyses.

No mammary tumors were found in castrate male mice receiving endocrine transplants at the age of 7 months. This confirms our earlier observations that there is with advancing age a loss of sensitivity of the mammary tissue to hormonal stimulation independent of the testicle.[6,7]

Summary. Syngenesiotransplants of ovaries made at one month of age caused mammary cancer in 5% of castrate male mice of strain A. The tumor incidence rose to 32.5%,

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if anterior hypophyses were grafted together with ovaries. Transplantation of anterior hypophyses alone failed to produce breast cancer. No mammary tumors occurred in castrates into which ovaries, anterior hypophyses alone or anterior hypophyses in combination with ovaries were grafted at 7 months of age. Thus in male mice, an age factor which is independent of the testicle operates in the production of breast cancer by ovarian and anterior hypophyseal grafts.

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Effects of Restricted Feed Intake in Intact and Ovariectomized Rats on Pituitary Lactogen and Gonadotrophin.*

JOSEPH MEITES AND JACK OWEN REED. (Introduced by F. W. Bernhart.)

From the Department of Physiology and Pharmacology, Michigan State College, East Lansing, Mich.

Starvation generally has been shown to result in decreases in the weights and functions of the endocrine glands, and these decreases have been attributed to a reduction in secretory activity by the anterior pituitary.[1–6] The adrenals represent an exception, since in cases of extreme or total starvation their weight and activity are actually augmented rather than reduced.[1,7,8]

Assays of the gonadotrophin content of the pituitaries of starved rats have revealed a decrease in the hands of some workers,[4,5] while others have reported no change in pituitary content.[9,10] The lactogenic hormone content of the pituitary was found to be reduced during complete starvation in lactating rats.[11]

It was the purpose of these experiments to seek further clarification of the effects of restricted food intake on the pituitary content of these two hormones, since under other conditions the latter has served as a reliable index of the amounts of these hormones present in the blood stream.[10–13] It was also considered desirable to determine the above effects in both intact and ovariectomized rats, since in the latter the pituitary gonadotrophin content is known to be higher[14–16] and pituitary lactogen lower than in intact rats.[13,17]

Methods. Forty-eight intact female rats of about 200 g body weight and 35 rats which had been ovariectomized about a month previous and weighed about 230 g were used in

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TABLE I. Weights of Endocrine Glands of Intact and Ovariectomized Rats on Restricted Feed Intakes.

No. rats per gp.	Procedure	Orig. body wt, g	Final body wt, g	% diff. in body wt	Ovarian wt per rat mg	Ovarian wt per 100 g body wt mg	Uterine wt per rat mg	Uterine wt per 100 g body wt mg	Adrenal wt per rat mg	Adrenal wt per 100 g body wt mg	Thyroid wt per rat mg	Thyroid wt per 100 g body wt mg	Pituitary wt per rat mg	Pituitary wt per 100 g body wt mg
	Intact Rats.													
10	Controls fed *ad lib.*	196	220	+12	62.4	28.4	393.4	179.0	63.2 ± 1.8*	28.8	18.1 ± 0.9*	8.2	15.2 ± 1.2*	6.9
10	Fed ¾ *ad lib.*	193	197	+ 2	52.3	26.6	374.5	190.4	54.0 ± 2.5	27.5	15.9 ± 0.9	8.1	11.7 ± 0.6	6.0
10	,, ½ ,, ,,	197	173	—12	50.5	29.1	256.0	147.7	47.3 ± 2.0	27.3	12.6 ± 0.6	7.3	10.9 ± 0.4	6.3
9	,, ¼ ,, ,,	200	153	—24	46.7	30.5	154.3	100.9	47.7 ± 3.0	31.2	13.0 ± 0.5	8.5	9.1 ± 0.2	6.0
9	No food	209	141	—33	51.1	36.2	184.0	130.5	61.8 ± 3.6	43.8	10.6 ± 0.2	7.5	8.9 ± 0.4	6.3
	Ovariectomized Rats.													
7	Controls fed *ad lib.*	230	248	+ 8					57.8 ± 3.0	23.1	21.9 ± 1.6	8.8	11.3 ± 0.8	4.6
7	Fed ¾ *ad lib.*	230	221	— 4					51.3 ± 1.9	23.2	16.1 ± 1.2	7.3	13.2 ± 0.7	5.9
7	,, ½ ,, ,,	228	194	—15					48.0 ± 3.3	24.7	15.8 ± 1.7	8.1	11.8 ± 0.5	6.1
7	,, ¼ ,, ,,	226	160	—30					52.3 ± 2.4	32.7	15.0 ± 1.1	9.4	11.1 ± 0.6	7.0
7	No food	234	162	—30					60.9 ± 1.6	37.6	16.8 ± 1.4	10.4	11.7 ± 0.5	7.2

* Standard error of the mean $= \sqrt{\dfrac{\Sigma d^2}{n(n-1)}}$

these experiments. All were of the Sherman strain. The intact and ovariectomized rats were divided into 5 groups each, and were put on *ad libitum*, ¾, ½, ¼ and no-feed regimes. The ¾, ½ and ¼ feed levels were determined daily from the *ad libitum* intake of the control group. The *ad libitum* and partially-fed groups were killed at the end of 14 days and the unfed group at the end of 7 days. The endocrine glands were removed, trimmed free of extraneous tissue, and weighed on a Roller-Smith balance.

The pituitaries from each group were macerated into a fine paste with a small mortar and pestle. Distilled water was then added to a volume of one ml per pituitary, and the fine suspension was taken up with a syringe through a number 25 needle.

Gonadotrophic potency was measured by injecting the equivalent of 5 pituitaries from each group into 5 immature female rats, making two injections daily for 5 days. On the 6th day the rats were killed and the ovaries and uteri were weighed. Lactogenic potency was assayed by injecting the equivalent of 2 pituitaries from each group intradermally over the crop glands of 5 mature White Carneau pigeons during a 4-day period, and rating the crop responses by the Reece-Turner[17] method on the 5th day.

Results. Although incidental to the main problem, the effects of restricted feed intake on the weights of the endocrine glands will be considered first (Table I). In the intact rats, the weights of the pituitary, thyroid, adrenals and ovaries were reduced on an individual basis in all the restricted diet groups, with the exception of the adrenal weights of the completely starved group. On a 100 g body weight basis, the weights of these glands remained the same, again with the exception of the adrenal weights of the completely starved group. These results agree on the whole with those previously reported by other workers.

In the ovariectomized rats, there was no loss of pituitary weight in any of the restricted diet groups; a slight but similar loss of thyroid weight in all restricted diet groups; and a slight loss of adrenal weight in all restricted diet groups except the non-fed group.

TABLE II. Assays of the Gonadotrophin and Lactogen Content of Pituitaries of Intact and Ovariectomized Rats on Restricted Feed Intakes.

Procedure	Avg pit. wt, mg	Body wt, g	Recipient rats* (gonadotrophin assays) Ovarian wt per rat	Ovarian wt per 100 g body wt	Uterine wt per rat	Uterine wt per 100 g body wt	Recipient pigeons‡ (lactogenic assays) Avg Reece Turner units per pit.	units per mg pit.
Intact Donor Rats								
Controls, fed *ad lib.*	15.2	77	18.6 ± 2.3†	24.2	53.3 ± 2.5†	69.5	4.5	0.30
Fed ¾ *ad lib.*	11.7	77	19.2 ± 2.5	25.0	71.9 ± 12.0	93.8	4.0	0.34
,, ½ ,, ,,	10.9	75	17.8 ± 2.2	23.8	72.4 ± 9.2	87.0	3.0	0.28
,, ¼ ,, ,,	9.1	78	20.0 ± 2.3	22.7	88.6 ± 3.9	92.5	2.5	0.27
No feed	8.9	81	17.8 ± 2.4	24.8	65.0 ± 9.1	110.0	2.2	0.25
Controls uninjected		74	15.2 ± 1.8	20.5	23.4 ± 4.6	31.0		
Ovariectomized Donor Rats								
Controls, fed *ad lib.*	11.3	68	65.7 ± 6.2	96.3	84.4 ± 5.0	123.8	3.1	0.27
Fed ¾ *ad lib.*	13.2	74	63.5 ± 11.8	85.8	116.0 ± 7.0	158.0	3.1	0.23
,, ½ ,, ,,	11.8	71	59.6 ± 7.2	84.1	85.0 ± 10.0	120.0	2.5	0.21
,, ¼ ,, ,,	11.1	73	65.6 ± 5.5	90.1	99.4 ± 5.4	136.5	1.8	0.16
No feed	11.7	72	65.7 ± 6.2	96.3	84.4 ± 5.0	123.8	1.8	0.15

* The equivalent of one donor rat pituitary was injected into each recipient rat.
‡ The equivalent of two donor rat pituitaries was injected into 5 pigeons.
† Standard error of the mean $= \sqrt{\frac{\Sigma d^2}{n(n-1)}}$

On a 100 g body weight basis, pituitary weight was increased in all restricted diet groups; thyroid weight was slightly reduced on the ¾ and ½ feed regimes but increased on the ¼ and no-feed regimes; and adrenal weight was unchanged on ¾ and ½ feed intake and increased on ¼ and no-feed intake.

These differential effects of restricted food intake on endocrine weights in intact and ovariectomized rats are interesting since they have not been reported previously. This may be due to the fact that almost all of the previous work on this problem has been done in intact animals. It is not known whether such differential effects would be obtained in intact and ovariectomized rats during more prolonged periods of feed restriction.

The effects of reduced feed intake on the lactogenic and gonadotrophic hormone content of the pituitaries are given in Table II. In neither the intact nor ovariectomized rats on restricted feed allowances was the gonadotrophin content of the pituitaries reduced from that of the controls fed *ad libitum*. In fact the concentration was actually increased on the basis of hormone content per milligram of pituitary in the intact groups (since pituitary weight was decreased on the restricted diets). The pituitaries of the ovariectomized rats contained about 10 times as much gonadotrophin as the intact rats, as indicated by their ability to increase ovarian weight in the recipient immature rats.

The lactogenic potency of the pituitaries of both intact and ovariectomized rats on reduced feed was decreased below that of the controls fed *ad libitum*, on an individual basis. The decrease in lactogenic hormone was roughly proportional to the decrease in feed intake. On the basis of hormone concentration per milligram of pituitary there was only a slight decrease in the intact groups and a marked decrease in the ovariectomized groups. In corroboration of previous findings[13,17] the lactogen content of the pituitaries of the ovariectomized control rats was reduced below that of the intact controls.

Discussion. Maddock and Heller[10] have suggested a possible cause for the divergent results reported by different workers on the assay of pituitary gonadotrophin content in starved rats. They pointed out that other investigators implanted entire pituitary glands into recipient assay rats, whereas they injected suspensions of pituitary tissue, as was also done in our experiments. The implantation method is believed to be less exact since necrosis, growth, or encapsulation may occur, resulting in an exceedingly variable delivery of gonadotrophic hormone.

It is not clear as to why the pituitary gonadotrophin content remains the same during periods of starvation while pituitary lactogen becomes reduced. There can be no doubt that starvation, depending on its degree, results either in a reduction or complete cessation of gonadal and mammary gland activity. This would certainly seem to indicate that the amounts of circulating gonadotrophic and lactogenic hormones are reduced.

On the assumption that pituitary content of a hormone represents the resultant between the amount being produced and the amount released into the blood stream, Maddock and Heller[10] explain the 'dichotomy' between gonadotrophin content in the pituitary and blood stream of starved rats as being due to a failure of release and to a minimum of production by the pituitary. The decrease of lactogenic hormone content in the pituitary during starvation could be explained as being due primarily to a failure of production with little or no interference with the release mechanism.

Assays of pituitary content of gonadotrophin have proven to be reliable indicators of the amounts present in the circulation following ovariectomy, during estrogen administration, but not during starvation. Assays of the lactogen content of the pituitary have accurately reflected the amounts present in the blood stream during lactation, during estrogen or testosterone administration, and also during periods of restricted feed intake.

Summary. The lactogenic and gonadotrophic hormone content of pituitaries of intact and ovariectomized rats was determined after full, ¾, ½, ¼ and no-feed regimes. The full or partially-fed groups were sacrificed at the end of 14 days and the unfed group at the end of 7 days. Pituitary lactogen content was reduced in the intact and ovariectomized rats on the ½, ¼ and no-feed regimes below that of the ¾ and full-fed controls. No change occurred in the gonadotrophin content of the pituitaries of either the intact or ovariectomized rats, regardless of the level of feed intake. In the intact but not in the ovariectomized rats, restricted feed intake caused a marked reduction in the weights of the pituitary, thyroid and adrenals, except that on the no-feed regime adrenal weight was increased in both groups.

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Assay of Secretin and Cholecystokinin Concentrates.*

LEON L. GERSHBEIN, C. C. WANG, AND A. C. IVY.

From the Department of Clinical Science, University of Illinois College of Medicine, Chicago

The most feasible assay of secretin concentrates involves the cannulation of the pancreatic duct and observation of the increased rate of pancreatic juice output upon intravenous injection of the sample. Ivy, Kloster, Drewyer and Lueth[1] introduced the threshold dose, namely, that amount of a secretin concentrate causing a flow of 10 drops (0.4 ml) of pancreatic juice within a 10-minute period over the control rate in the dog. In a later work, Greengard and Ivy[2] reported that the threshold dose of a preparation obtained from hog duodenums (SI) and adopted as a standard was 0.25 mg for many dogs. This

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value was employed for assay in conjunction with the ratio of weights of unknown to standard eliciting a 10 drop response in 10 minutes. Still[3] determined secretin unitage from the ratio of drops of unknown to a vasodilatin-free standard produced by the injection of 4 mg of each. Neither sample data nor comments as to reproducibility were given. Penau and Simonnet[4] derived a unit from uniform injections of secretin concentrates and measurement of juice output after 15, 30 and 45 minutes. A high variability resulted. A marked departure from the above direct methods is that of Wilander and Ågren[5] who employed narcotized cats. A unit was arrived at from the titration of the alkalinity of secretions from the duodenum soaked up by a rolled-up filter paper. Among others, a criticism of this method is that the alkalinity is not due to pancreatic juice alone. A comparison of this cat unit with the one obtained by Greengard and Ivy has been made.[2,6]

A direct method for the assay of cholecystokinin (CCK) fractions described by Ivy and Oldberg[7] involves cannulation of the gall bladder after clamping the cystic duct. Contractions were observed by connection to a recording tambour. A unit of CCK potency was defined as that amount of vasodilatin-free concentrate which when injected intravenously brings about a gall bladder contraction of one centimeter of bile. Recently[8] a method has been reported for CCK estimation which involves the recording of an increase in the pressure in the common bile duct after the injection of CCK in unanesthetized dogs, the common bile duct being cannulated through a duodenal fistula. The *in vitro* contraction of guinea pig[9] and frog[10] gall bladders has been used for assaying CCK preparations. However, numerous difficulties are encountered with this method, notably, the regulation of intravesical pressures. In the present report, details are given for the assay of secretin and CCK, both of which may be carried out on the same dog. New reproducible units of potency are derived for these hormones by comparison of the gall bladder or pancreatic response produced by the standard with that of a test concentrate.

Preparation of the Standard Concentrate. A representative batch of SI prepared by treatment of a hog duodenal extract with trichloroacetic acid according to the method of Greengard and Ivy[2] was adopted as the standard. Although a few products proved to be somewhat more potent, the standard concentrate was chosen on the basis of the absence of vasodilatins. The dry standard has been stored at room temperature for over 2 years with no detectable loss in potency. A freshly prepared aqueous or dilute hydrochloric acid solution is used in the assay.

Assay Procedure. Dogs, 9-13 kg in weight, which have been fasted for at least 19 hours are placed under pentobarbital anesthesia. The main pancreatic duct is cannulated and after filling of the cannula, a rubber or plastic tube containing saline is connected so as to register juice output on a drop recorder. Carotid blood pressure is recorded throughout. A priming dose of secretin concentrate is injected into the exposed femoral vein, and, after some time, the basal rate of flow is determined. This is useful in the exploration of a practical drop rate especially where a pancreas is hypersecretive. A known amount of standard SI is injected and the number of drops of pancreatic juice, which decreases rapidly in rate with time, is recorded over a 10-minute period. After basal conditions prevail, the test concentrate is then administered in an amount yielding the same drop count within one to two drops over the same time interval. The ratio of standard to test sample dosages is determined. Further samples may be assayed with frequent checking of the standard dosage.

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For the assay of cholecystokinin, the cystic duct is clamped or ligated after isolating it from the cystic artery, and a grooved metal cannula is secured in the gall bladder. A glass tube of 8 mm outside diameter is connected between the cannula and the recording tambour. Where an insufficient amount of fluid is present in the glass tube, additional bile is added. The standard SI is injected and the gall bladder contraction, which is complete within the first few minutes, is recorded in millimeters by noting the initial and final bile levels in the glass tube. When virtually the same initial level is restored, the test extract is introduced in such dosage that the same rise is obtained within 1 ml; however, for a rise of 10 mm or more, 2 mm are often allowed in the comparison, depending on the sensitivity of the gall bladder. The ratio of standard to test dosages is then determined. Since the response to the standard often varies with time and the condition of the dog, frequent or alternate checking is necessary.

It is important in the comparison of a test sample with the standard to treat both in a similar manner especially as regards the initial bile-pressure level, and readings must be taken with consistency particularly in the case of hypersensitive gall gladder preparations. For assay purposes, it was found that more accurate results are obtained by measuring the liquid rise in the glass tube than by comparing the respective heights on the tambour tracing. Secretin and CCK assay values can be checked by employing a comparison at a different drop rate or gall bladder contraction, respectively. The use of derived standards or concentrates of known potency repeatedly checked against the SI standard is also of value.

Since the presence of vasodilatins in a concentrate can have a marked effect on the secretin and CCK assay results, the blood pressure recording must be closely followed. Where additional pentobarbital is administered, sufficient time should elapse before the animal is restandardized.

Variation of Gall Bladder Response with CCK Dosage. A study of the response of the gall bladder to varying doses of CCK concentrates was carried out on several dogs. For some of the large contractions, time intervals up to 45 minutes were required before initial conditions were restored. The majority of the data was rechecked.

Results. Sample data typical for the secretin assay are shown in Table I for 6 preparations carried out on a number of dogs. The respective ratios did not change on comparison of the standard and the test concentrate at a different common drop rate. The value of a respective potency ratio is taken as the number of units of secretin contained in 1 mg of the concentrate. Table II illustrates the potency ratios or CCK unitage per mg for 5 fractions with good reproducibility in almost all cases. The concentration of the standard SI solution was usually 1 mg per ml. Although more potent concentrates were diluted so that the injected volumes of test and standard solutions were similar in many cases, no significant deviations resulted when they differed.

CCK Dose-Response Relationships. By plotting the respective responses or gall bladder contractions against gradually increasing doses of CCK, curves were obtained of the type given in Fig. 1. Except for curve III obtained with a concentrate of 2.0 units of CCK per mg, standard SI was employed.

Discussion. The results of the assay methods outlined for secretin and CCK show clearly that reproducible ratios are obtained which are independent of the weight of the test animal. Furthermore, where the condition of the dog may change after the determination of a ratio, restandardization often gives the same value. This discovery renders the determination of an Ivy secretin threshold dose less reliable than the method herein described. As can be seen from the fluctuation of dosage for a given drop rate from one dog to another in Table I, the former method of assay required at least 10 dogs for an acceptable assay.

The values of the potency ratios are equated to unitage of secretin or CCK contained in 1 mg of the concentrate. The results could be equally well expressed in multiples of these

TABLE I.
Assay Values for Secretin Preparations.

	Dog No.	Preparation, mg	SI standard, mg	Pancreatic juice, drops	Secretin potency ratio (units secretin/mg)
Prep. S-61	16	1.0	2.5	12	2.5
	17	1.3	3.0	9	2.3
	51	.81	2.0	28	2.5
	57A	.46	1.1	11	2.4
	58	.75	1.8	38	2.4
	59	1.3	3.2	37	2.5
	67	1.25	3.0	11	2.4
Prep. S-14	1	2.0	3.0	10	1.5
	16	1.5	2.5	9	1.7
	17	2.7	4.0	10	1.5
	25	3.1	4.5	14	1.5
	47	1.95	2.9	10	1.5
	48	2.8	4.2	25	1.5
	49	1.25	2.0	14	1.6
	51	1.3	2.0	15	1.5
	58	1.2	1.8	27	1.5
	61	4.0	6.0	11	1.5
Prep. S-75	1	1.5	3.0	10	2.0
	12	5.0	10.5	9	2.1
	47	1.3	2.6	14	2.0
	51	1.0	2.0	25	2.0
	54	.60	1.2	9	2.0
	55	1.2	2.4	10	2.0
	56	.50	1.05	40	2.1
	57A	.55	1.1	16	2.0
	58	.90	1.8	27	2.0
	59	1.6	3.2	50	2.0
Prep. S-73	12	2.0	10.5	10	5.3
	25	.80	4.5	19	5.6
	26	.95	5.0	10	5.3
	40	.075	0.40	14	5.3
	41	.28	1.5	17	5.4
	47	.50	2.7	8	5.4
	51	.38	2.0	12	5.3
	59	.60	3.2	40	5.3
	64	.56	3.0	40	5.4
	65	.40	2.2	30	5.5
	67	.50	2.8	21	5.6
Prep. S-165	26	.39	5.0	12	13
	36	.32	4.0	9	12.5
	41	.080	1.0	9	12.5
	53	.12	1.5	13	12.5
	58	.14	1.8	27	13
	68	.16	2.0	28	12.5
	71	.20	2.5	33	12.5
	72	.23	3.0	10	13
	73	.25	3.0	17	12
	74	.40	4.8	27	12
Prep. S-36	66	.25	7.6	15	30
	67	.10	3.0	11	30
	70	.12	3.6	20	30
	72	.10	3.0	10	30
	73	.10	3.0	19	30
	76	.10	3.0	32	30
	77	.10	3.0	20	30
	83	.075	2.2	12	29

TABLE II.
Assay Values for CCK Concentrates Compared Against Standard SI.

	Dog No.	Preparation, mg	SI standard mg	Gall bladder contraction, mm	CCK potency ratio (units CCK/mg)
Prep. C-4	16	4.0	6.5	10	1.6
	25	3.1	4.5	11	1.5
	40	.50	.75	12	1.5
	47	1.9	2.4	8	1.3
	48	2.7	4.2	7	1.6
	49	1.25	2.0	15	1.6
	50	1.25	2.0	8	1.6
	51	1.3	2.0	10	1.5
	58	1.2	1.8	23	1.5
	61	4.0	6.0	6	1.5
Prep. C-7	9	4.5	9.0	9	2.0
	37	8.0	16.5	9	2.1
	47	1.3	2.6	9	2.0
	49	.40	.80	8	2.0
	51	1.0	2.0	10	2.0
	54	.60	1.2	10	2.0
	55	1.2	2.4	10	2.0
	56	.50	1.05	8	2.1
	57A	.55	1.1	5	2.0
	58	.90	1.8	20	2.0
Prep. C-60	15	3.5	9.0	10	2.6
	50	.84	2.3	5	2.7
	51	.75	2.0	10	2.7
	56	.39	1.0	7	2.6
	58	.73	1.8	24	2.5
	67	1.25	3.0	20	2.4
Prep. C-37	12	1.2	5.0	10	4.2
	16	1.5	6.5	10	4.3
	25	.90	4.5	11	5.0
	40	.090	.40	10	4.4
	47	.50	2.2	7	4.4
	51	.46	2.0	9	4.3
	64	.70	3.0	11	4.3
	67	.70	3.0	17	4.3
	68	.70	3.0	19	4.3
Prep. C-100P	152	.30	3.5	11	12
	161	.46	5.5	10	12
	170	.60	7.5	35	12.5
	175	.20	2.5	22	12.5

values or in terms of any arbitrary unitage or percentage. Although comparison of test fractions can also be affected by reference to more potent standards, SI has been chosen on the basis of its uniformity of preparation and the presence of both secretin and CCK.

Any method for the biological assay of secretin is fraught with difficulties. For unknown causes, some dogs are very insensitive to secretin. In some cases, after a small dose of secretin is injected the increase in the flow of juice is sustained for longer than 10 minutes. Also, in the case of the CCK assay, it must be remembered that a smooth muscle response is involved. Aside from difficulties arising from the inability of separating the cystic artery and trauma to the gall bladder, some preparations are quite insensitive and, in some cases, the initial conditions of intra-gall bladder pressure are not attained. Only well-functioning gall bladder preparations should be employed for the assay of concentrates. A large number of these difficulties are minimized by employing the present method of comparing the response of test concentrate with that of the standard.

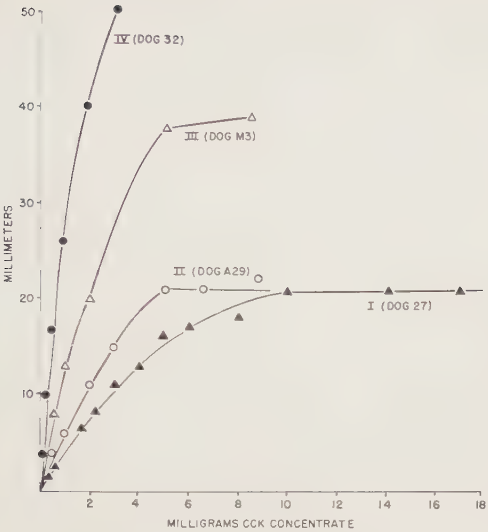

Fig. 1.

Response of the gall bladder to varying doses of cholecystokinin. Curves I, II and IV represent Standard SI; III was obtained for Preparation C-7 containing 2.0 units CCK/mg.

As might be predicted, the administration of increasing doses of CCK brings about gall bladder contractions which increase until a maximum is attained, and beyond which there is no further increase in response in spite of further doses of CCK. This maximum contraction which corresponds to complete emptying of the viscus, varies from one dog to another and is definitely affected by the volume of the gall bladder. Actually, in the CCK assay, volume changes are involved and well-filled gall bladders are preferable. In the comparison of the test sample with the standard, a contraction lower than the maximum must be employed, and this can be checked by noting if a greater bile-pressure rise occurs upon increasing this dosage. Large contractions are avoided, since often a longer time is required for the attainment of initial conditions.

The dose response curve for secretin has been shown by Greengard, Stein and Ivy[11] to be S-shaped in character, and Burn and Holton[12] have used the lower linear portions for the comparison of secretin concentrates with their standard which is comprised of an acetone-dried duodenal powder. Since a maximum in drop response is obtained with elevated doses of secretin, comparisons must be made at drop counts below this limiting value. It should also be pointed out that the log dose-response curves, which are linear for CCK and secretin in the region far removed from their respective maxima, could also be applied to the assay of concentrates. However, it is relatively simple to determine the requisite potency ratio in subsequent dogs by the method outlined in this paper after a first value has been obtained. Usually 3 dogs are sufficient as can be noted in Tables I and II.

Summary. The assay of secretin and CCK concentrates are affected by cannulation of the pancreatic duct and the gall bladder, respectively. The ratio of dosages of a vasodilatin-free standard concentrate (SI) to the test fraction yielding the same number of drops of pancreatic juice in a 10-minute interval is determined, and the value is taken as the secretin unitage contained in 1 mg of test fraction. Similarly, the dosage ratio of this standard to the unknown giving the same gall bladder contraction is equated to the units of CCK per mg of unknown. Good reproducibility is obtained in a large number of dogs.

The dose-response curves for CCK and their significance are discussed.

The authors are grateful to Dr. R. W. Denton for aid in surgical techniques, and to Mr. Max Krup for independent checking of a number of the assays.

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16978

Anaerobic Replacement of Carbon Dioxide.*

Samuel J. Ajl and C. H. Werkman.

From the Bacteriology Section, Iowa Agricultural Experiment Station, Ames, Iowa.

Previous reports[1] on the heterotrophic replacement of carbon dioxide in bacteria have dealt exclusively with aerobic metabolism, largely because the compounds substituting for CO_2 were known to occur in the normal aerobic metabolism of bacteria. Further research in this field, however, reveals first that organisms normally requiring CO_2 when growing aerobically require it anaerobically, and secondly, that the same compounds replace CO_2 under anaerobic conditions. The purpose of the present communication is to report on the anaerobic replacement of carbon dioxide and to compare the function of these compounds under aerobic and anaerobic conditions.

Methods. The methods used were essentially those employed in previous work.[1] The organisms used in these experiments were *Escherichia coli* and *Aerobacter aerogenes.* The inoculum consisted of a 24-hour culture grown in 10 ml of the basal medium consisting of 0.8% K_2HPO_4, 0.4% $(NH_4)_2SO_4$, 0.8% glucose and 10% tap water, final pH adjusted to 6.8. The compounds tested were added before autoclaving except in the case of oxalacetic acid. A solution of the sodium salt was sterilized by filtration and added aseptically.

Cylinder nitrogen was passed over hot reduced copper gauze to remove oxygen and then through a CO_2-absorbing train before passing through the cultures. The reaction flask contained 25 ml of basal medium to which 2% of inoculum was added aseptically. Growth was measured by turbidimetric readings on a Klett-Summerson photoelectric colorimeter with a 660 mμ light filter. All readings were made after 12 hours incubation at 37°C. Sterile, uninoculated media were used as blanks.

Experimental. Neither *E. coli* nor *A. aerogenes* will grow anaerobically in the absence of CO_2. Atmospheric CO_2 can be replaced by $NaHCO_3$; these results are in accord with Gladstone *et al.*[2] Since no growth takes place in the absence of CO_2, the conclusion may be drawn that CO_2 has a definite function under anaerobic conditions.

The compounds and the extent to which they replace CO_2 are listed in Table I. The results are essentially similar to those obtained aerobically with the exception of oxalacetic acid which most effectively replaces CO_2 in the complete absence of oxygen. It is, therefore, possible that oxalacetic acid is a key compound in the anaerobic metabolism of the cells.

α-Ketoglutaric acid (and glutamic acid) substitutes anaerobically to a greater extent than any of the other compounds, except oxalacetate. Similar results were obtained aerobically. These facts again suggest a possible further fixation over and above the Wood and Werkman reaction. Such a fixation reaction would in normal metabolism (in the presence of CO_2) yield C_5 acids which are more essential to the cell. Indirect evidence for this has been obtained. More abundant growth is obtained with bicarbonate and succinic, malic or fumaric acid than with any of these acids alone (Table II).

A number of compounds metabolically related to those of Table II will also replace CO_2 anaerobically (Table III).

In the case of *A. aerogenes,* the function of citric acid under anaerobic conditions should be stressed. Whereas, the effect is comparatively small aerobically, in the absence of O_2 the organism uses this acid very effectively

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* Journal Paper No. J-1199 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 862.

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TABLE I.

Effect of Addition of Various Compounds on Anaerobic Growth of *E. coli* in the Absence of CO_2.

C_3-Compounds	Growth	C_4-Compounds	Growth	C_5-Compounds	Growth	C_6-Compounds	Growth
dl-Alanine	6	Succinic Acid	30	α Ketoglutaric Acid	85	Citric Acid	9
Pyruvic Acid	4	Fumaric Acid	40	Glutamic Acid	200	cis-Aconitic Acid	25
		Malic Acid	35				
		Oxalacetic Acid	220				
		Aspartic Acid	62				

All compounds were used in 2-mM quantities. Growth is indicated by turbidimetric readings on the photoelectric colorimeter.
Control experiments give readings from 3 to 9.
Total volume, 25.5 cc. Incubation time, 12 hr. Temp., 37°C

TABLE II.

Effect of Added Carbonate on the Growth of *E. coli* in the Presence of C_4 Compounds.

Compound	With $NaHCO_3$	Without $NaHCO_3$
Succinic	55	30
Fumaric	65	40
Malic	60	45

All of the compounds including $NaHCO_3$ were added in 2-mM quantities. The extent of growth is indicated by turbidimetric readings on the photoelectric colorimeter.

Control gave readings from 3 to 9.

Total volume, 25.5 cc. Incubation time, 12 hr. Temp., 37°C.

in the place of CO_2 (Table IV). *E. coli* will not utilize citric acid under the above conditions.

The fact that the compounds normally occurring in the Krebs cycle replace CO_2 anaerobically suggests a possible function of this cycle in the absence as well as in the presence of oxygen. However, it should be noted that the existence of this cycle has not been demonstrated in bacterial respiration.

It is generally considered that the tricarboxylic acid cycle is an aerobic mechanism by which various substrates can be oxidized to CO_2 and H_2O, to yield energy to the system. Since the final products of anaerobiosis are not generally the same as in the presence of O_2, some other function of this cycle must be proposed. Since fat, protein, and carbohydrate metabolism have many points in common in the form of identical intermediate products and since the compounds supplied in the absence of CO_2 are some of the interconvertible intermediate products, it is possible that by amination, transamination, and other reactions the various compounds are directly utilized for the synthesis of proteins. On such a basis the utilization of CO_2 to form these compounds which in turn are used for cellular material can then be explained.

Summary. A number of compounds have been found which will replace CO_2, necessary for the anaerobic metabolism of *Escherichia coli* and *Aerobacter aerogenes*. The C_5 compounds appear to replace CO_2 more effectively than other compounds tested with the exception of oxalacetic acid and citric acid, the latter in the case of *A. aerogenes* only. The

TABLE III.
Effect of Glutamine, Asparagine, Arginine, and Proline on Anaerobic Growth of *E. coli* in the Absence of CO_2.

Compound	Glutamine	Asparagine	Arginine	Proline	Control
Growth	125	40	55	30	5

The extent of growth is indicated by turbidimetric readings on the photoelectric colorimeter.
Compounds were added in 2-mM quantities.
Total volume, 25.5 cc. Incubation time, 12 hr. Temp., 37°C.

TABLE IV.
Replacement of CO_2 by Citric Acid Under Aerobic and Anaerobic Conditions.

	Citric Acid	
Organism	Nitrogen with O_2 and CO_2 removed	Air with CO_2 removed
Escherichia coli	9	4
Aerobacter aerogenes	180	50

The extent of growth is indicated by turbidimetric readings on the photoelectric colorimeter.
2-mM quantities of citric acid were used.
Total volume, 25.5 cc. Incubation time, 12 hr. Temp., 37°C.

fact that oxalacetic by-passes the CO_2 requirement to such an extent indicates that this acid may function as the chief substrate for protein synthesis under anaerobic conditions.

16979

Magnesium and Hyaluronidase Inhibitor of Blood Serum.

MONROE E. FREEMAN, RICHARD WHITNEY, AND ALBERT DORFMAN.*

From the Department of Chemistry and Physics, Army Medical Department Research and Graduate School, and the Department of Pediatrics, University of Chicago Medical School.†

Numerous reports[1-12] have established the presence of a hyaluronidase inhibitor in the blood sera of several species. The nature of the inhibitor has not been elucidated; but evidence has been presented that it is not a specific antibody;[2,13-16] nor an enzyme specifically attacking hyaluronidase.[6,9,12] The

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* Address: Department of Pediatrics, University of Chicago Medical School.

† Contribution from University of Chicago carried out under contract with Medical Research and Development Board, Surgeon General, U. S. Army.

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TABLE I.
Hyaluronidase Inhibitor Activity of Human Blood Serum Fractions in the Presence of Different Amounts of Magnesium Ion.

	No added magnesium		Additional magnesium	
	mM of Mg ion per ml reaction mixture, × 10^{-5}	Hyaluronidase inhibitor units per ml serum	mM of Mg ion per ml reaction mixture, × 10^{-5}	Hyaluronidase inhibitor units per ml serum
Serum A	4.4	70	68	150
Component IA	.8	7	60	28
" IIA	.8	5	60	75
" IA and IIA	.8	10	60	100
Residue	2.9	—	—	—

inhibitor may be a polysaccharide or protein-polysaccharide complex successfully competing with the hyaluronic acid as an alternate substrate.[3,7,12]

Partial separation of serum into two fractions that were inactive alone, but active when recombined, seemed to indicate that the inhibition was probably more complicated than originally supposed. Both fractions were first reported to be of protein nature:[9] Component I was flocculated from serum at -2°C, pH 8.4, and 25% ethanol; followed by component II flocculated by lowering the pH to 6.1. Another report[10] showed that citrated, oxalated, and dialyzed serum was not active unless magnesium was added. Studies in these laboratories indicated that when human blood serum was fractionated according to method of Cohn *et al.*,[17] much of the activity appeared in fractions II and III; but only if the fraction was dissolved in a solution containing magnesium ions.

Further experiments on the fractionation of normal human serum with respect to the hyaluronidase inhibitor and the contribution of magnesium to the activity of the inhibitor are herewith reported.

Methods. Twenty frozen stored samples of human blood sera were selected at random from a series originally obtained from young adult males. The hyaluronidase, from frozen bovine testis, was prepared, assayed, and standardized by previously described methods.[18,19] Sera were assayed for hyaluronidase inhibitor by incubating suitable dilutions with standardized hyaluronidase, subsequently assaying the reaction mixture for active hyaluronidase.[12] Dilutions were made with saline-borate buffers with and without measured amounts of magnesium chloride. Magnesium content of the undiluted serum and its fractions was determined with titan yellow.[20] Thus, the relative amounts of serum and magnesium present in the reaction mixture could be calculated and the relation expressed as millimoles of magnesium per ml of reaction mixture. The inhibitor activity was calculated as units of hyaluronidase inactivated per ml of serum.[19] Increased amounts of magnesium were added to diluted samples of serum until 4 or 5 successively higher magnesium concentrations gave no further increase in inhibitor activity.

Normal human serum was buffered to pH 8.4 with barbiturate, cooled to 0°C, and component I was flocculated by the slow addition of an equal volume of 50% ethanol. After separating and washing the precipitate in a refrigerated centrifuge, the supernate was brought to pH 6.1 with 0.6 *M* acetate buffer, pH 4.0. The precipitate that formed, component II, was removed and washed in refrigerated centrifuge. The two precipitates were suspended in volumes of 0.15 *M* saline equal to the volume of the original serum and

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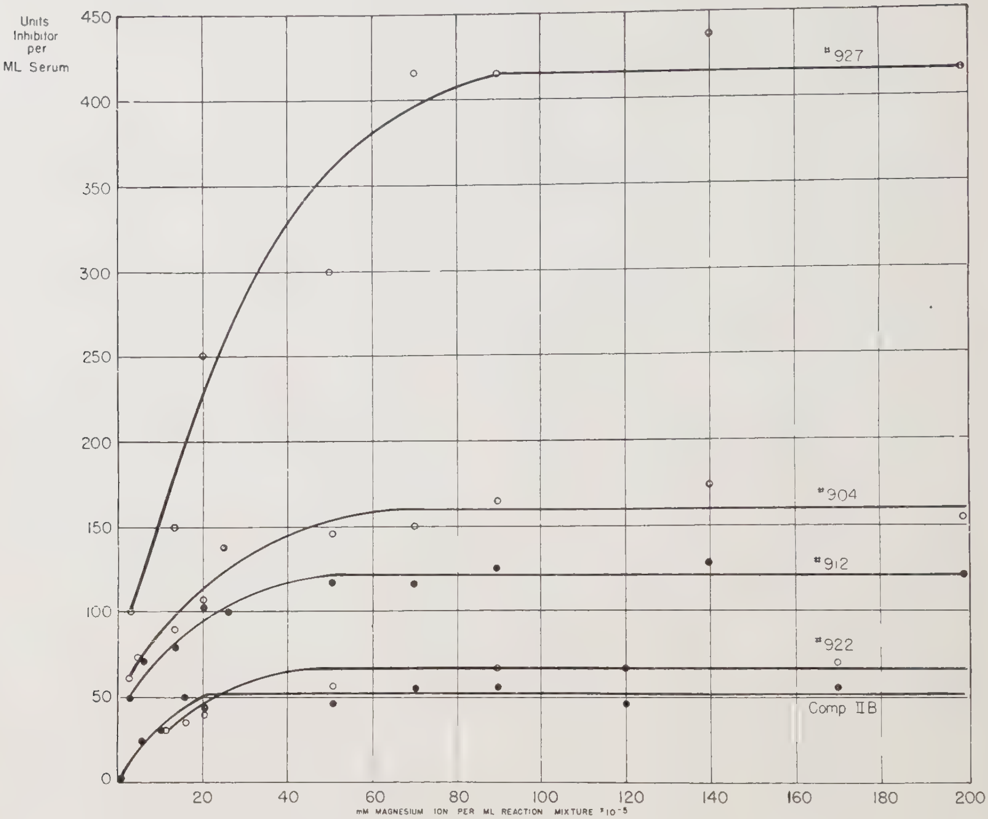

FIG. 1.
Relation of magnesium concentration to inhibitor activity in assay reaction mixture.

assayed for hyaluronidase inhibitor with and without additional magnesium. Serum components and supernate were analyzed for magnesium and assayed for inhibitor with the results shown in Table I as units of inhibitor per ml of serum and millimoles of magnesium per ml of the reaction mixture.

Table I illustrates the values found in 3 fractionations of normal human sera. The original sera contained approximately 80.10^{-5} millimoles of magnesium ion per ml. This is 18 times the magnesium ion content of the assay reaction mixture after suitable dilution of the serum with saline. This reaction mixture assayed 70 inhibitor units per ml of serum. In order to obtain the maximum inhibitor activity of 150 units per ml of serum, it was necessary to increase the magnesium concentration of the assay mixture to at least $60 + 10^{-5}$ millimoles per ml by diluting the serum with saline-magnesium solutions instead of saline alone.

The washed precipitates of component I contained small amounts of magnesium; less than one-fifth of the total serum magnesium. It was apparent that the precipitates contained insufficient magnesium when suitably diluted with saline for assay. When these precipitates were diluted with saline containing sufficient magnesium to support maximum inhibition, only a part of the total inhibitor was found.

Component II precipitates also contained minimum amounts of magnesium and when diluted with saline alone gave little indication of inhibitor activity. When diluted with saline-magnesium solution, however, these precipitates gave inhibitor values equal to at least half of the total activity of the serum. When components I and II were mixed, the

TABLE II.
Hyaluronidase Inhibitor Activity of Blood Sera With and Without Added Magnesium.

	Normal blood serum		Added magnesium	
Sample	mM of Mg ion per ml reaction mixture, $\times 10^{-5}$	Hyaluronidase inhibitor units per ml serum	Total mM of Mg ion per ml reaction mixture, $\times 10^{-5}$	Hyaluronidase inhibitor units per ml serum
925	5.0	43	65	75
920	3.5	24	66	87
917	4.1	40	68	110
929	4.1	50	67	112
918	4.1	50	68	116
916	5.2	47	68	117
934	4.1	73	67	145
919	3.5	71	68	148
921	3.3	63	68	150
932	4.1	78	67	150
923	4.2	62	68	165
926	4.5	63	68	167
924	5.0	75	68	175

saline dilutions were again very low in magnesium; and consequently, showed little activity. When sufficient magnesium was added, the activities of the mixtures were greatly enhanced.

These experiments clearly indicated that the hyaluronidase inhibitor in human serum and in the active serum fractions required some minimum concentration of magnesium ion for expression of its maximum activity *in vitro*. The actual amounts of magnesium required for maximum activity were determined by experiments illustrated in Fig. 1. These data demonstrated that when sera, or its active fractions, were diluted with saline alone, very low inhibitor activities were found in the assay reaction mixtures. Increasing amounts of magnesium in the reaction mixture were accompanied by regular increments of activity until a maximum activity was reached where the reaction mixture contained about 50 to 60 x 10^{-5} millimoles of magnesium ion per ml. Further increases or decreases in activity were within the experimental error of the assay up to concentrations of 200 x 10^{-5} millimoles.

It should be noted, however, that the assay method employed demanded an inhibitor concentration of only 2 to 4 units per ml in reaction mixture. This restriction necessitated a 20 to 100-fold dilution of most sera. Thus, when the serum was diluted with saline alone, the magnesium concentration, about 80 x 10^{-5} millimoles per ml of serum, fell to less than 5 x 10^{-5} millimoles per ml of the reaction mixture. Consequently, low inhibitor values resulted. Maximum inhibitor values were found when the magnesium concentration was increased to 60 x 10^{-5} millimoles per ml, very near that of the original serum. This was clearly shown in Fig. 1 where data were plotted for component IIB and 4 sera of high, low, and intermediate activity.

Thirteen additional samples were determined by diluting the sera with saline alone, and by diluting with saline containing adequate concentrations of magnesium. These data, showing the necessity of magnesium in the inhibitor assay, are given in Table II.

Summary. Magnesium ion has been found to be essential for the hyaluronidase inhibitor activity of human blood under the conditions of the *in vitro* assay. The minimum amount of magnesium required for expression of maximum activity has been determined for the assay method employed in these laboratories.

16980

Lack of Effect of Sodium Phenosulfazole (Darvisul) on Certain Experimental Virus Infections.*

Gerald A. LoGrippo, David P. Earle, Jr., Bernard B. Brodie, Irving P. Graef, Robert L. Bowman, and Robert Ward.

From the Departments of Medicine and Pediatrics, New York University College of Medicine and Bellevue Hospital, New York, N.Y.

Sodium phenosulfazole, or Darvisul, (N-(2-thiazolyl)-phenol sulfonamide)† has recently been described by Sanders, SubbaRow and Alexander[1] as protecting mice against as many as 100 LD_{50} doses of the Columbia SK murine virus.

The present report is concerned with tests of Darvisul against the MM virus,‡ the Lansing strain of poliomyelitis virus, and Theiler's virus of mouse encephalomyelitis (TO strain). No protective effect was found.

CFW mice (Carworth Farms) 5 to 6 weeks old, weighing 15 to 20 g were used in all but one experiment. Eight mg of Darvisul in 1 ml of physiologic saline were given twice daily by the intra-abdominal route. In the experiment on Theiler's virus (Table II) 3 to 4 week old mice were used conforming to our standard procedure with TO virus and the drug was fed in the diet.

In Experiment 1 (Table I) the procedure described by Sanders and his associates[1] was followed precisely, *e.g.* 8 mg of Davisul were given twice daily beginning 24 hours after intra-abdominal injection of MM virus and continued for 5 days. No significant difference was observed in the treated and control animals. In Experiments 2 and 3, Darvisul was begun 2 days before and continued for 6 days after infection. The suggestive difference obtained in Experiment 2 was not reproduced in Experiment 3. In Experiments 4 and 5, MM virus was given by a route different from that of Darvisul—subcutaneously and by mouth, respectively. No appreciable effect was noted. The *in vitro* effect of Darvisul on MM virus was tested in Experiment 6. Instead of inactivation of virus, the titer of the suspension of virus plus Darvisul was more than 100 times greater than the titer of the virus control. In Experiment 7, Darvisul was begun 2 days before and continued for 6 days after intracerebral injection of the Lansing strain of poliomyelitis virus. The results were negative. Experiment 8 is concerned with Theiler's virus of mouse encephalomyelitis as found in the intestines of normal mice (Table II). The capacity of Darvisul to reduce the amount of TO virus excreted in the feces was tested. The stools of individual mice were assayed for virus before and after drug administration. In this experiment a 2% drug diet was fed to 3 mice for 7 days. The amount of Darvisul consumed was 72 to 88 mg per day. In contrast to certain other compounds tested in this laboratory,[4] Darvisul had no effect upon the excretion of TO virus in the feces of these mice.

Summary. Under the circumstances which have been described, Darvisul revealed neither prophylactic nor therapeutic value in mice infected with the MM virus, the Lansing strain of poliomyelitis virus or Theiler's virus of mouse encephalomyelitis (TO).

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* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

† This compound was made available through the courtesy of Lederle Laboratories Division, American Cyanamide Co., Pearl River, N. Y.

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‡ MM virus has been shown to be closely related serologically to Columbia SK virus and the virus of encephalomyo-carditis.[2,3]

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TABLE I.
Lack of Effect of Darvisul on MM Virus and Lansing Poliomyelitis Virus Infection in Mice.

Exp. No.		Darvisul Dose, mg/day	Darvisul Drug route	Infection Virus route	Infection Virus Strain	Conc. of virus 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	LD_{50} titer	Remarks
1	Control	0	—	IP	MM			15/15*	15/15	14/14	11/12		$10^{-6.5}$ or more	Drug admin. begun 24 hr after infection and continued 5 days.
	Treated	16	IP	”	”			15/15	14/15	13/13	9/14		$10^{-6.2}$ or more	
2	Control	0	—	”	”				10/10	9/10	4/10		$10^{-5.7}$	Drug admin. 2 days pre and 6 days post infection.
	Treated	16	IP	”	”				7/9	2/10	1/9		$10^{-4.6}$	
3	Control	0	—	”	”				10/10	7/10	2/10	1/10	$10^{-5.5}$	Ditto No. 2.
	Treated	16	IP	”	”			10/10	10/10	7/10	5/10	1/10	$10^{-5.8}$	
4	Control	0	—	SC	”					9/9	8/10	4/10	$10^{-6.7}$	Ditto No. 2.
	Treated	16	IP	”	”					9/9	7/10	2/9	$10^{-6.1}$	
5	Control	0	—	oral	”		4/9	1/8						Ditto No. 2.
	Treated	16	IP	”	”		6/10	6/10						
6	Control	*In vitro*		IP	”		6/6	6/6	4/6	0/6	0/6		$10^{-4.2}$	1% virus vs. 1% drug at 37°C for 2 hr after which dilutions were made with distilled water.
	Treated	exper.		”	”		6/6	6/6	6/6	6/6	5/6		$10^{-6.4}$ or more	
7	Control	0	—	IC	LA	7/8	4/8	0/8	0/8				$10^{-1.7}$	Ditto No. 2.
	Treated	16	IP	”	”	7/7	2/8	0/8	0/8				$10^{-1.7}$	

* 15/15 Numerator = number of mice paralyzed; denominator = number of mice inoculated. Mice dying without symptoms have been excluded. IP = Intraperitoneal; IC = Intracerebral; SC = Subcutaneous; LA = Lansing poliomyelitis virus.

TABLE II.
Lack of Effect of Darvisul on the Excretion of Theiler's Virus (TO).

Exp. No.	Darvisul Individual mice treated	Darvisul Average dose drug/day, mg	Assay of TO virus in feces of individual mice Before Darvisul	Assay of TO virus in feces of individual mice After 7 days of Darvisul
8	Mouse No. 1	88	8/10*	8/10
	” ” 2	74	2/9	1/7
	” ” 3	72	10/10	9/9

* 8/10 = 8 out of 10 mice became paralyzed following inoculation of feces from mouse No. 1.

16981

Failure of Phenosulfazole (Darvisul) in Treating Experimental Viral Infections.

HERALD R. COX, HILARY KOPROWSKI, ARDEN W. MOYER, GEORGE R. SHARPLESS, AND SAM C. WONG.

From the Section for Viral and Rickettsial Research, Lederle Laboratories Division, American Cyanamide Company, Pearl River, N. Y.

Recently Sanders, SubbaRow and Alexander[1] reported sodium phenosulfazole (Darvisul), N-(2-thiazolyl)-phenol sulfonamide, to be effective against at least as many as 100 LD_{50} doses of the Columbia SK virus[2,3] for mice when intraperitoneal treatment was instituted 24 hours after intraperitoneal infection.

The purpose of this communication is to report the failure of phenosulfazole to protect mice and monkeys against experimental infection with the Columbia SK virus, and the Brunhilde strain of poliomyelitis virus, respectively, as well as its lack of effect against certain other viral infections in mice and in chick embryos.

A. Experiments with Columbia SK Virus. Exp. 1. Groups of 15 mice (Rockland Swiss albino), weighing 20 to 24 g each, were inoculated intraperitoneally with graded doses of Columbia SK virus (a frozen pool of infected mouse brains received through the courtesy of Dr. Murray Sanders) and treated with phenosulfazole by the same route. Treatment was started 24 hours after virus inoculation and continued for 6 consecutive days. The phenosulfazole used in this experiment was obtained from Dr. Sanders as a 25% solution and represented a portion of the same preparation that was used in his laboratory. The drug was diluted with sterile saline solution to give a concentration of 8.26 mg per ml and given at 8 and 11:30 a. m., and 3 and 5 or 6 p. m. in doses of 0.5, 0.25, 0.25 and 1.0 ml respectively. Thus, the dose per day per mouse was 16.5 mg. Control mice were injected in a manner similar to the treated group except that sterile physiological saline solution was used. The mice were observed for 21 days at which time the mortality ratios were calculated.

Exp. 2 This experiment was similar to Exp. 1 except that slightly smaller mice (Rockland Swiss albino), about 18 to 20 g in weight, were used. Dilutions of the Columbia SK virus, 10^{-4} through 10^{-7}, were used with 20 mice per each dilution. Group 1 mice received 22 mg phenosulfazole* per mouse per day. Group 2 mice received 32 mg of drug per mouse per day. Group 3 mice served as virus controls and received injections of sterile saline solution in place of the drug. Group 4 mice received no virus but served as drug controls and received 22 mg phenosulfazole per day. Group 5 (only 10 mice in this group) likewise received no virus but received 32 mg phenosulfazole per day as drug controls.

Treatment was started 20 hours after infection and continued for 12 days. The drug was used in a concentration of 20 mg per ml. The mice received 4 injections (intraperitoneally) per day. Those of Group 1 and 4 received 6 mg at 8 a. m., 4 mg at 11:30 a. m., and 3 p. m., and 8 mg at 5 p. m., whereas Groups 2 and 5 received 8 mg each at 8 and 11:30 a. m., and 3 and 5 p. m. The mice were

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[1] Sanders, M., SubbaRow, Y., and Alexander, R. C., *Texas Reports Biol. and Med.*, 1948, **6**, 385.

[2] Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1940, **72**, 407.

[3] Committee on Nomenclature of the National Foundation for Infantile Paralysis, A Proposed Provisional Definition of Poliomyelitis Virus, *Science*, 1948, **108**, 701.

* The phenosulfazole used in this and most of the subsequent experiments was received from Dr. M. C. Lockhart, Director of the Pharmaceutical Section, Lederle Laboratories Division, American Cyanamide Company, as a sterile 25% solution in sealed glass ampoules.

TABLE I.
Results Obtained with Phenosulfazole in the Treatment of Mice Infected with Columbia SK Virus.

Exp. No.	Wt of mice, g	Drug per day, mg	Mortality ratio† of mice infected with virus dilutions				LD_{50} titer
			10^{-4}	10^{-5}	10^{-6}	10^{-7}	
1	20-24	16.5	15/15	13/15	9/15	12/15	$10^{-7.0}$
		0* (Virus control)	12/15	12/15	9/15	11/15	$10^{-6.6}$
2	18-20	22	16/20	12/20	4/20	7/20	$10^{-5.4}$
		32	14/19	12/20	7/20	9/20	$10^{-5.6}$
		0* (Virus control)	10/19	9/20	8/20	4/20	$10^{-5.0}$
		22 (No virus drug control)	0/20				
		32 (No virus drug control)	0/10				

* Received injections of buffered saline solution instead of drug.
† Numerator = No. of mice dying; denominator = No. of mice in group.

observed for 16 days at which time the mortality ratios were calculated.

The results of the first two experiments are summarized in Table I. The data show that phenosulfazole had no beneficial therapeutic effect in mice infected with the Columbia SK virus and treated as described above. In addition there was no evidence to suggest that the drug affected the onset or duration of the infection.

B. Experiments with Poliomyelitis Virus in Monkeys. Exp. 3. Five monkeys (*M. rhesus*) were infected intranasally under ether anesthesia with 4 ml each of a 10% suspension of a frozen pool of monkey cords infected with the Brunhilde strain of poliomyelitis virus (3rd passage in this laboratory of material received from Dr. Isabel Morgan, Baltimore, Md.). Three monkeys were treated with phenosulfazole whereas 2 served as virus controls and received sterile saline solution in place of the drug. Treatment was started 24 hours after infection and consisted of 200 to 300 mg intravenously at 8 a. m., 300 mg intraperitoneally (intravenously on one occasion) at 12 noon, and 500 mg intraperitoneally at 5 p. m. Treatment was continued for 11 days. The results may be summarized by stating that no evidence was obtained to indicate that treatment with phenosulfazole showed any beneficial effect.

The 3 treated monkeys reacted as follows: No. 26 showed definite weakness of both arms and legs on the 5th day, complete paralysis of both legs on the 8th day, was prostrate on the 9th day, and was sacrificed in a moribund condition on the 10th day. No. 34 showed tremors and weakness of both legs and arms on the 10th and 11th day, paralysis of the left arm and leg on the 12th and 13th day, extreme weakness with inability to move from the 14th to 23rd day but from thereon gradually improved and eventually recovered with some residual paralysis. No. 43 showed progressive weakness from the 11th through the 13th day, was paralyzed in both arms and legs on the 14th day and was sacrificed in a moribund condition on the 15th day.

The 2 untreated monkeys reacted as follows: No. 9 showed no signs of illness throughout the 28 days of observation, whereas No. 42 developed paralysis of both legs and arms on the 15th day and was sacrificed in a moribund condition on the 16th day.

C. Experiments with some Neurotropic Viruses and Influenza Virus in Mice. Three experiments were carried out with the MEF-1 strain of poliomyelitis virus (obtained through the courtesy of Dr. Peter K. Olitsky of the Rockefeller Institute for Medical Research, New York, N. Y.), using 50 to 500 LD_{50} doses inoculated intracerebrally into Rockland Swiss albino mice. In Exp. 1 treatment of the infected animals was initiated 24 hours after infection. In Exp. 2, in which 2 separate groups were treated, drug therapy was started 24 hours after infection in one group, and 6 hours after infection in the other. In Exp. 3, two groups of treated animals were again employed with the drug being administered 6 hours after infection in one group, and 1½ hours after infection in the other.

Two experiments were carried out with Eastern equine encephalomyelitis virus using Tumblebrook Swiss albino mice. In one case the mice were inoculated by the intracerebral route and in the other by the intraperitoneal route. In both cases drug therapy was initiated 24 hours following infection.

Two experiments were carried out using Venezuelan equine encephalomyelitis virus inoculated subcutaneously into Rockland Swiss albino mice. In both experiments drug treatment was started 24 hours following infection.

A single experiment was carried out with Russian Spring-Summer encephalitis virus inoculated subcutaneously into Carworth Swiss albino mice. Drug therapy was started 24 hours following infection.

Two experiments were carried out with the PR8 strain of influenza virus (Type A) inoculated intranasally into Rockland Swiss albino mice. In the first experiment drug therapy was started 24 hours following infection whereas in the second it was started 1½ hours following infection.

The essential data pertaining to these experiments are shown in Table II. The results may be summarized by stating that again no evidence was obtained to show that phenosulfazole had any beneficial therapeutic effect in mice infected with any of the above named viral infections.

D. Experiments in Chick Embryos. A number of experiments were carried out to determine the effect of phenosulfazole upon chick embryos infected with Newcastle disease virus, the Lee strain (Type B) of influenza virus, the Bitter Root strain of Rocky Mountain spotted fever (*R. rickettsii*), the Breinl strain of epidemic or louse-borne typhus (*R. prowazekii*) and the 6 BC strain of psittacosis (*M. psittacii*). The experiments may be summarized by stating that only in the case of psittacosis, in which relatively large doses of the drug were employed (25 mg per embryo), was there any suggestive evidence that phenosulfazole had a protective effect. This was demonstrated by the fact that drug-treated embryos showed a slight increase in their survival time and a reduction in the number of elementary bodies observed by microscopic examination of yolk-sac smears, as compared to infected control eggs treated with saline in a similar manner. This was not an unexpected finding, however, because other sulfonamides, particularly sulfadiazine, have been shown to be active against psittacosis.[4-6] It should be pointed out, however, that the results obtained with phenosulfazole in the treatment of chick embryos infected with *M. psittacii* were not nearly as satisfactory as those reported recently for the antibiotic Aureomycin.[7]

Conclusion. Phenosulfazole (Darvisul) showed no beneficial effect in the treatment of mice infected with the Columbia SK virus and other neurotropic viruses, including a rodent adapted strain of poliomyelitis (MEF-1 strain). The lack of therapeutic effectiveness of the drug was also observed in monkeys infected intranasally with the Brunhilde strain of poliomyelitis virus. In other viral and rickettsial infections of mice and of developing chick embryos treatment with phenosulfazole was of no therapeutic value, with the possible exception of psittacosis in which a slight inhibition of growth in the developing chick embryos was observed. However, even in the latter case the effectiveness of phenosulfazole therapy was of much lower magnitude than that observed with other sulfonamides,[4-6] or antibiotics.[7]

ADDENDUM

Since this paper was written an additional experiment was carried out using Columbia SK virus in Carworth Swiss albino mice in a manner as identical as possible to that em-

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[4] Meiklejohn, G., Wagner, J. C., and Beveridge, G. W., *J. Immunol.*, 1946, **54**, 1.

[5] Wiseman, R. W., Meiklejohn, G., Lackman, D. B., Wagner, J. C., and Beveridge, G. W., *J. Immunol.*, 1946, **54**, 9.

[6] Rosebury, T., Ellingson, H. V., and Meiklejohn, G., with assistance of Schabel, F., *J. Infect. Dis.*, 1947, **80**, 64.

[7] Wong, S. C., and Cox, H. R., *Ann. N.Y. Acad. Sc.*, 1948, **51**, 290.

TABLE II.

Results Obtained with Phenosulfazole in the Treatment of Mice Infected with Other Viruses.

Virus inoculum	Route of infection	Exp. No.	Wt of mice, g	Drug per day, mg	Mortality ratio* of mice infected with virus dilutions 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	LD_{50} titer
Poliomyelitis MEF-1	I.C.	1	10-12	10	10/12								
				0†	6/6								
		2	10-12	16	10/10	8/10							
				16‡	10/10	10/10							
				0†	9/10	9/10							
		3	10-14	8‡	12/12								
				8§	12/12								
				0†	12/14								
Eastern Equine Encephalomyelitis	I.C.	1	14-16	20						6/6	2/5	3/6	$10^{-7.4}$
				0†						6/6	6/6	0/6	$10^{-7.5}$
	I.P.	2	14-16	20				3/6	4/6	1/6	0/6		$10^{-4.7}$
				0†				1/6	1/6	0/6	0/6		$<10^{-4.0}$
Venezuelan Equine Encephalomyelitis	S.C.	1	20-22	4				6/6	6/6	5/5	6/6	6/6	$>10^{-8.0}$
				0†						6/6	0/6	0/6	$10^{-6.5}$
		2	20-22	8				6/6	6/6	5/6	3/6	3/6	$10^{-7.3}$
				0†				6/6	6/6	4/6	1/6	1/6	$10^{-6.4}$
Russian Spring-Summer Encephalitis	S.C.	1	18-20	20			4/4						
				0†‖			4/4						
Influenza PR-8	I.N.	1	16-18	22					8/9				
				0†					8/9				
		2	16-18	22§					9/9				
				0†					8/9				

* Numerator = No. of mice dying; denominator = No. of mice in group.

† Untreated controls.

‡ Drug treatment started 6 hr after virus inoculation.

§ Drug treatment started 1½ hr after virus inoculation.

‖ The 10^{-3} dilution of Russian Spring-Summer encephalitis contained 25 to 100 mouse LD_{50}.

ployed by Sanders *et al.*[1] Groups of 25 to 28 mice, weighing 16-20 g each, were inoculated intraperitoneally with 0.06 ml each of graded doses of SK virus ranging from 2×10^{-4} to 2×10^{-8}. Treatment was initiated 24 hours after virus inoculation and continued for 5 consecutive days. Group 1 mice received 1 ml of the drug intraperitoneally (8.33 mg phenosulfazole per ml) at 8 a.m. and 6 p.m. Group 2 mice were injected in similar manner with sterile saline solution, while Group 3 mice received no treatment. The LD_{50} titers were as follows: Group 1 (phenosulfazole treated) $10^{-6.3}$, Group 2 (saline treated) $10^{-6.6}$, Group 3 (no treatment) $10^{-6.1}$.

16982

Therapeutic Failure of Phenosulfazole (Darvisul) in Mice Infected with EMC or with MM Viruses.

Marvin L. Weil and Joel Warren.

From the Department of Virus and Rickettsial Diseases, Army Medical Department Research and Graduate School, Army Medical Center, Washington, D.C.

Phenosulfazole (Darvisul) has been reported by Sanders, SubbaRow, and Alexander[1] as having a therapeutic effect in mice infected with "a mouse virus isolated in 1940." Elsewhere in their report[1] the authors mention "SK mouse poliomyelitis," hence they presumably worked with the Columbia-SK virus which is a neurotropic agent immunologically indistinguishable from the encephalomyocarditis (EMC) and MM viruses.[2] The lack of effect of this drug on the course of the disease in mice experimentally infected with EMC and MM viruses is reported here. The phenosulfazole used in the present work was supplied by Dr. H. R. Cox of the Lederle Laboratories.

In Exp. 1, groups of 30 Swiss mice (Bagg strain) weighing 12 g each were infected with varying amounts of EMC virus intraperitoneally and treated with phenosulfazole by the same route. The drug was diluted to contain 6.25 mg/cc and given at 8 and 11 a. m. and 2 and 5 p. m. in doses of 0.5, 0.25, 0.25 and 1.0 cc respectively. Treatment was begun 17 hours after infection and continued for seven days with a total daily dose of 12.5 mg. Control mice were injected in the same manner as the treated group except that normal saline was used. Following infection the mice were observed for 17 days when the death ratios were calculated. It should be noted that few deaths occurred subsequent to the ninth day after infection. Selected animals in the treated and control groups were autopsied and the virus recovered by passage of brain tissue into normal mice.

In Exp. 2, groups of 16 mice weighing about 20 g each were infected and treated in a manner similar to that employed in Exp. 1, except that MM virus was used and the schedule of therapy was varied somewhat. Therapy was initiated in the 3 groups of treated mice as follows: (a) 2 hours before infection (b) 2 hours after infection and (c) 19 hours after infection. In each instance the initial dose was 8.33 mg followed by a course of treatment similar to that described above to give 16.7 mg daily. Because of evidence of toxicity the daily dosage was reduced to 12.5 mg per mouse on the second day and was maintained at this level until stopped 5 days after infection. It should be noted that in this experiment the titer of the MM virus in the control mice was at least $10^{-7.9}$. That the upper limit of infectivity could not have been much beyond this range is indicated by the

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[1] Sanders, M., SubbaRow, Y., and Alexander, R. C., *Texas Rep. Biol. and Med.*, 1948, **6**, 385.

[2] Warren, J., and Smadel, J. E., *Fed. Proc.*, 1948, **7**, 311.

TABLE I.
Phenosulfazole in the Treatment of Mice Infected with EMC or with MM Viruses.

Virus	Therapy		Dilution of infectious material				MLD 50%
	Initial dose (hr)	mg/day	$10^{-5.0}$	$10^{-6.0}$	$10^{-7.0}$	$10^{-8.0}$	
EMC	0 (control)		30/30*	21/29	17/30	5/30	$10^{-7.0}$
	17 post infection	12.5	30/30	29/30	23/30	—	$10^{-7.3}$†
MM	0 (control)		16/16	12/16	12/16	11/16	$10^{-7.9}$†
	2 pre inf.	12.5‡	13/14	14/15	11/14	—	$10^{-7.3}$†
	2 post inf.	12.5‡	14/14	14/16	10/14	—	$10^{-7.2}$†
	19 post inf.	12.5‡	16/16	15/16	9/16	—	$10^{-7.1}$†

* Numerator = No. of mice dying; denominator = No. of mice in group.

† These figures represent minimal values. The exact titers are at least this great.

‡ Initial dose 8.33 mg/mouse. These mice received 16.7 mg drug per day during the first 24 hours and 12.5 mg per day subsequently. See text.

fact that this same preparation of frozen stored virus when titrated five days later had a titer of $10^{-7.5}$.

The results of the two experiments are summarized in Table I. These data indicate that treatment with phenosulfazole produced no reduction in mortality among the mice infected with either EMC or MM viruses. Furthermore, there was no indication that it delayed the onset or duration of the disease.

16983 P

Evaluation of the Effect of Darvisul upon Infection with SK Strain of Virus in Mice.*

Thomas Francis, Jr., and Gordon C. Brown.

From the Department of Epidemiology and Virus Laboratory, School of Public Health, University of Michigan, Ann Arbor, Mich.

Darvisul, N-(2-thiazolyl)-phenol sulfonamide, or phenosulfazole was reported by Sanders, SubbaRow, and Alexander[1] to be effective against as many as 100 LD_{50} of a mouse virus, SK, when administration was begun 24 hours after intraperitoneal infection.

Through the courtesy of Dr. Sanders the virus was received in the form of two infected mouse brains in glycerine. The drug was received in ampules of 25% solution of lot No. 7-8522, through the kindness of Dr. Sanders and Dr. Leo Rane of the Lederle Laboratories. The plan of testing the drug was that outlined by Dr. Sanders and was rigidly observed.

The original brain material was made into a 10% suspension in 10% horse serum physiological salt solution. A titration was done by intraperitoneal inoculations of groups of 5 mice and found to be approximately 2×10^{-7}. A pool was made from the brains of mice sacrificed when moribund and the 10% suspension of this material served as the material with which the therapeutic effects of the compound were tested.

Albino mice of 20 g weight of a single strain were used. As shown in the table 50 mice were inoculated intraperitoneally with the respective concentrations of virus and 24 hours later administration of the chemical was begun by the same route. The stock solutions of compound were diluted 1:31 in physiological salt solution so that each ml contained 8 mg. The material was given

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* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Sanders, M., SubbaRow, Y., and Alexander, R. C., *Texas Rep. Biol. and Med.*, 1948, **6**, 385.

TABLE I.
Evaluation of Effect of Darvisul on SK Virus in Mice.

Dilution of virus	Treated No. survivors		Controls No. survivors	
	5th day	14th day	5th day	14th day
2×10^{-4}	0/25	0/25	0/25	0/25
2×10^{-5}	1/25	0/25	1/25	0/25
2×10^{-6}	7/25	2/25	7/25	2/25
2×10^{-7}	21/25	18/25	23/25	18/25

Date of Experiment = October 3, 1948.
Numerator = survivors. Denominator = total number inoculated.

intraperitoneally to 25 mice in amounts of 0.5 ml at 9 a.m., 0.25 ml at 1 p.m., 0.25 ml at 4 p.m., and 1.0 ml at 7 p.m. so that the total daily dosage was 16 mg. Control mice, 25 in number, received equivalent volumes of salt solution intraperitoneally.

The results are presented in Table I. It is apparent that no difference between treated and control mice was observed. It is concluded, therefore, that under the conditions of the experiment Darvisul had no therapeutic effect upon the infection of mice with the SK strain of murine encephalomyelitis virus.

16984

Behavior of the Thyroid toward Elements of the Seventh Periodic Group. I. Halogens and Thiocyanates.*

Emil J. Baumann and Nannette Metzger.

From the Laboratory Division, Montefiore Hospital, New York City.

It will perhaps surprise many readers of this report to learn that the thyroid will filter from the blood not only iodine but the other halogens as well. Further, while it is generally known that feeding thiocyanates to man and other animals will cause the development of goiter, thyroid hyperplasia and goiter have also been produced by feeding relatively large amounts of any of the halogens other than iodine. The evidence for these statements will be assembled, much of it from the literature, and an hypothesis offered that attempts to show that bromides, chlorides, fluorides, as well as thiocyanates all bring about thyroid enlargement by the same means, and to explain why the responses of the thyroid to all of these substances are similar.

Filtration of Halogens and Thiocyanates by the Thyroid. The first step in formation of thyroxine consists in filtering iodide from the blood. This filtration by the thyroid is remarkably effective, its concentrating power being of the order of 10,000. In an early experiment of Marine and Feiss[1] nearly 90% of a dose of 5 mg of KI was found in the goitrous thyroid of a dog in 10 minutes. In many more recent experiments with radioactive iodine similar results have been noted. Even when the synthesis of thyroxine is prevented by administration of thiourea and related substances, the thyroid can still filter iodine from the blood, first shown by Baumann, Metzger, and Marine[2] and subsequently by others. Selective filtration by the thyroid is not limited to iodine; in fact the gland behaves in a similar fashion with all the elements of the 7th group of the periodic table that have been studied, *viz.*, Cl, Br. Mn, and At, as well as with thiocyanates. It may be predicted that fluorine, rhenium, and technetium (element 43) will be found to behave in the same way.

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* A report of this work was made before the American Society of Biological Chemists at Chicago, May 14, 1947, *Fed. Proc.*, 1947, **6**, 237.

[1] Marine, D., and Feiss, H. O., *J. Pharm. Exp. Therap.*, 1915, **7**, 557.

[2] Baumann, E. J., Metzger, N., and Marine, D., *Endocrinol.*, 1944, **34**, 44.

Baumann, Sprinson, and Marine[3] found more Br in rabbit thyroids than in other tissues, especially when the gland was hyperplastic, an observation that had been made by many other investigators. Ray and Desach,[4] after injecting subcutaneously small amounts of $MnCl_2$ daily for 6 days, noted an accumulation of Mn in all tissues, but they found that the thyroid had a concentration 2 to 10 times as great as any other tissue studied. And somewhat similar experiments were made with Astatine by Hamilton and Soley[5] using the tracer technic. This element, prepared by α particle bombardment of Bi by Corson, Mackenzie, and Segrè[6] is radioactive. It was found to behave as a metal rather than like the halogens, *e.g.*, it is not precipitated by $AgNO_3$ from nitric acid solution while it is precipitated by H_2S from an acid solution. Hamilton and Soley fed tracer doses of this isotope to guinea pigs with normal or with hyperplastic thyroids. They found many times more Astatine in the thyroid than in any other tissue they examined.

Experimental. We compared the concentration of Cl^- and SCN^- in thyroid and other tissues of albino rats. Both normal and goitrous animals were used. The goitrous rats were fed our stock food mixture to which 1% thiouracil[†] was added for 2 to 5 weeks. The tissues of two or more rats were pooled and treated as follows:

Finely minced tissues were analyzed for Cl^- by digestion over a free flame with 5 ml standard $AgNO_3$ and a few drops $KMnO_4$ if necessary (Eisenman[7]). For thiocyanate determination the finely minced tissues were weighed into 25 or 50 ml Erlenmeyer flasks and digested with 10-20 ml 4% alcoholic KOH in a boiling water bath. To limit evaporation a small funnel covered with a watch glass was placed in each flask while heating. After half an hour, the funnel and cover glass were removed, the contents of the flask evaporated to dryness, neutralized with 5 N H_2SO_4, adding 0.2 ml in excess and finally 1.5 ml 10% Na_2WO_4. After the precipitated protein had flocked out, the mixture was made up to 10 ml, centrifuged and filtered. A 75-80% aliquot was analyzed by the method of Baumann, Sprinson, and Metzger.[8]

Adult rats were used to measure the Cl^- content of tissues. Our stock diet consisting of mixed cereals, dried milk, yeast, meat scrap, and salts contains about 1.3% NaCl. Due to the special role of chlorides in the physiology of tissue fluids, one would hardly expect to find as much Cl^- in thyroid as in blood. However, thyroid did contain 2 or 3 times as much Cl^- as muscle while the Cl^- concentration of tissues having much blood, such as liver, was only slightly less than in thyroid. Some of the data of these tests are given in Table I.

In the thyiocyanate experiments adult rats were injected intraperitoneally with 2 ml of a 5% NaSCN solution and sacrificed by chloroform anesthesia 4 to 5 hours later. Blood was taken from the heart, the tissues dissected and prepared for analysis. Some of these data are given in Table II. A similar experiment was made on a 2.5 kg rabbit in which 7 ml of 5% NaSCN were injected intraperitoneally. This proved to be irritating, the rabbit showing signs of discomfort for about 15 minutes, but no hyperemia of the peritoneum was found at autopsy 5 hours later.

It will be seen from Table II that thyroid tissue always contained a greater concentration of SCN^- than any other tissue, though

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[3] Baumann, E. J., Sprinson, D. B., and Marine, D., *Endocrinol.*, 1941, **28**, 793.

[4] Ray, T. W., and Desach, J., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 228.

[5] Hamilton, J. G., and Soley, M., *Proc. Nat. Acad. Sci.*, 1940, **26**, 483.

[6] Corson, D. R., MacKenzie, K. R., and Segrè, E., *Phys. Rev.*, 1940, **57**, 459.

[†] We thank the American Cyanamid Company, who supplied the thiouracil.

[7] Eisenman, A. J., *J. Biol. Chem.*, 1929, **82**, 411.

[8] Baumann, E. J., Sprinson, D. B., and Metzger, N., *J. Biol. Chem.*, 1934, **105**, 269.

TABLE I.
Comparison of Cl^- Concentration in Thyroid and in Other Fresh Tissues.

No. rats	Treatment	Thyroid Cl, mg per g	Muscle Cl, mg per g	Pancreas Cl, mg per g	Liver Cl, mg per g
3	Normal	1.48	.50		1.26
5	,,	1.19	.47		1.11
5	,,	1.30	.50	1.20	1.07
5	,,	1.27	.49	1.05	1.12
7	,,	1.29	.51	1.00	1.13
4	,,	0.75	.44		
2	Thiouracil	0.81	.43		
2	,,	1.30	.47		1.03
2	,,	1.32	.46		0.98
2	,,	1.99*	.44	1.30	1.12
2	,,	1.56*	.95		1.27

* These rats had snuffles-pneumonia.

TABLE II.
Comparison of Concentration of SCN^- in Thyroid and in Other Fresh Tissues.

No. animals	Thyroid, wt, g	Thyroid SCN, mg per g	Muscle SCN, mg per g	Liver SCN, mg per g	Blood SCN, mg per g	Kidney SCN, mg per g
2 rats	.209	.49	.21	—	.30	.43
2 ,,	.147	.64	.18	—	.31	—
2 ,,	.119	.71	.48	.42	.53	.66
3 ,,	.259	.73	.51	.38	.40	.62
3 ,,	.213	.70	.37	.40	.58	.66
2 ,,	.125	.80	.60	.50	.54	.74
Rabbit	.179	.59	.30	.29	.26	.37

the kidney which is the excretory organ for thiocyanate had only a slightly lower concentration than the thyroid. The level of tissue SCN^- was largely determined by the elapsed time between injection and death.

The daily intake of iodine necessary to maintain a normal thyroid in an albino rat is only 2 or 3 μg. If 50 to 250 mg of halides are added daily to the food of rats, a not very excessive amount, they would receive 16,000 to 80,000 times as much halide as iodide. And since all halogens are selectively filtered by the thyroid one could reasonably expect the added halide to have a mass action-like effect on the iodine normally ingested, thus preventing it from reaching the thyroid. Such is the case. Experiments have been made with fluoride, chloride, bromide, and thiocyanate (which behaves like the halogens) and with each of these substances, thyroid hyperplasia was produced.

Thus Goldemberg[9] found that rats given NaF for several months develop very hyperplastic thyroids, as much as 8 times the normal size. Hibbert[10] produced thyroid hyperplasia in rats on low iodine intake by giving 2% $CaCl_2$ or 3% NaCl. These observations were confirmed and extended by Remington[11] who fed diets containing 1 to 4% of NaCl. The thyroid iodine was reduced to one-third of the amount in the control animals. Morruzzi[12] and Simon[13] found on continued feeding of NaBr to young rats that thyroid I was decreased and goiter developed. The many more recent experiments in which goiter has been produced by giving thiocyanates to man and other animals are well known and need not be specifically cited.

Our observations and those from the literature are summarized in Table III. Table III indicates (1) that the thyroid selectively

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[9] Goldemberg, L., *C. R. soc. biol.*, 1926, **95**, 1169.

[10] Hibbert, J. S., *Arch. Surg.*, 1933, **26**, 648.

[11] Remington, R. E., PROC. SOC. EXP. BIOL. AND MED., 1938, **37**, 652.

[12] Morruzzi, G., *Boll. soc. ital. biol. sper.*, 1940, **15**, 733.

[13] Simon, I., *Boll. soc. ital. sper.*, 1941, **16**, 562.

TABLE III.
Summary of Thyroid Response to Administration of Group VII Salts.

	Selectively filtered by thyroid	Reduces thyroid iodine	Produces thyroid hyperplasia and goiter
F	?	?	+
Cl	+	+	+
Br	+	+	+
I	+	—	—
SCN	+	+	+
Mn	+	?	?
At	+	?	?

filters from the blood SCN^-, the halides, and other members of group VII of the periodic table. This inference is based on the greater concentration of these ions found in the thyroid compared with other tissues; (2) that the reduction of thyroid iodine is brought about by giving 0.1 to 0.4 g SCN^-, F^-, Cl^-, Br^- to rats daily for several weeks (or greater doses to larger animals); and (3) that the development of thyroid hyperplasia follows as a consequence of the lowering of thyroid iodine.

Discussion. The investigations mentioned above as well as others have all been concerned with but one of these ions. There has been no very satisfactory explanation offered of how any of them act. In fact most workers have been content to go no farther than to present their findings. For example Wolff, Chaikoff, Taurog, and Rubin[14] in studies on thiocyanate goiter concluded that SCN^- inhibited the uptake of I by the thyroid, but no explanation of the inhibition was offered. In a study of the uptake and discharge of I by propylthiouracil and thiocyanate-induced goiters, VanderLaan and Bissell[15] found that SCN^- did interfere with the uptake of I but this could be prevented by giving large amounts of I simultaneously. Propylthiouracil, on the other hand, inhibited thyroid hormone synthesis but did not interfere with the "trapping" mechanism, an effect similar to that described by Baumann, Metzger, and Marine[2] for thiourea a number of years before. This study was extended by J. E. and W. P. VanderLaan[16] who found that SCN^- inhibited the preferential accumulation of small amounts (<1.0 mg) of iodide and that this inhibition persisted for 5 hours. Furthermore they found that bromides in a dose 500 times greater than that of the injected iodide will not cause significant I displacement from the thyroid. In this experiment apparently a single dose of bromide was used though no details are given. It does not necessarily conflict with those of Morruzzi[12] and of Simon,[13] for the latter fed bromides continuously for months and uniformly found thyroid iodine greatly diminished under these conditions.

In all the thyroid-halogen-SCN^- work so far considered, massive doses of halide or thiocyanate have almost always been used, many hundreds or thousands of times as much as the I ingested. We believe that these anions exert a mass action-like effect; as a consequence inorganic iodide is washed out of the thyroid and new supplies of I are prevented from being taken up. The iodine present as thyroxine and diiodotyrosine is used up in the course of a few weeks, and since new supplies of I cannot be received, thyroid hyperplasia and goiter develop as soon as the thyroid I falls below a critical level. Not only do the halides and SCN^- behave this way toward I, but we[3] have found that large doses of chlorides remove bromides from the thyroid. We believe that continued administration of relatively large amounts of any halide will wash out any other halide present in small amount.

Thiocyanates behave in the same way as do the halogens. For they too accumulated preferentially in the thyroid and at the same time washed iodide out, thus producing thyroid hyperplasia. This similar behavior of SCN^- is not surprising; rather it is to be expected from the position of SCN^- at the end of the Hofmeister anion series (acetate$>$ $Cl^->Br^->I^->SCN^-$, etc.) Since the effects of these anions on protein swelling, surface tension, and on many other phenomena increase as the SCN^- end of the series is approached, it is reasonable to suppose that the ability of the thyroid to filter these ions from the blood would be affected similarly.

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[14] Wolff, J., Chaikoff, I. L., Taurog, A., and Rubin, L., *Endocrinol.*, 1946, **39**, 140.

[15] VanderLaan, W. P., and Bissell, A., *Endocrinol.*, 1946, **39**, 157.

[16] VanderLaan, J. E., and VanderLaan, W. P., *Endocrinol.*, 1947, **40**, 403.

A question that arises is how the thyroid differentiates between the halogens and other ions. A point of similarity is found in the arrangement of their electrons. All have 7 in their outer shells consisting of 2 in the s and 5 in the p groups. We suggest that this characteristic electron arrangement of the halogens is one that may enable thyroid tissue to effect this differentiation. At the same time it would explain why *all* halides are filtered from the circulating medium in preference to other ions. While thiocyanates closely resemble the halides in their chemical properties, and while the behavior of tissues towards halides and thiocyanates is similar we do not know whether their nuclear structure is correspondingly alike.

Further to support this view one should note the preferential accumulation of Mn and At by the thyroid. Like the halogens these elements of the seventh periodic group also have 7 electrons in their outer shells but instead of 2 s and 5 p electrons, the arrangement is believed to be reversed. And as stated in the first part of this paper one would also expect the remaining elements of group VII, *viz.*, technetium (element 43), and rhenium, to be preferentially filtered from the blood.

It becomes a matter of great interest to determine the relative avidity of the thyroid for these elements, to determine whether the preference follows a definite order, and to note the effect of the reversal of the electrons in the outer shell.

Summary. 1. Thyroid filters preferentially and accumulates thiocyanates as well as all the elements of the seventh periodic group that have been studied, *viz.*, Cl, Br, I, Mn, At.

2. All halides and thiocyanates when administered in relatively large amounts compared with iodine wash inorganic iodide from the body and prevent new supplies of iodine from being received by the thyroid, thus producing thyroid hyperplasia and goiter. It is suggested that these ions exert, at least in part, a mass action-like effect.

3. It is further suggested that similarity in arrangement of electrons in the outer shell of the elements of the seventh periodic group enables the thyroid to differentiate between them and other elements.

16985

Water Content of Rat Uterus and Oviducts During Proestrus and at End of Heat.*

D. LOUISE ODOR AND RICHARD J. BLANDAU. (Introduced by C. E. Tobin.)

From the Department of Anatomy, University of Rochester School of Medicine and Dentistry.

During the period of sexual receptivity in the rat the uterine cornua are distended with fluid which is retained by the tonic contractions of the cervix.[1,2] The appearance of the fluid within the lumina coincides with a significant decrease in uterine tissue water.[3] Also during heat several of the ampullar loops of the oviducts become greatly distended with

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* This investigation was supported by a grant from the Committee for Research in Problems of Sex, National Research Council.

[1] Long, J. A., and Evans, H. M., *Mem. Univ. Calif.*, 1922, **6**, 1.

[2] Blandau, R. J., *Am. J. Anat.*, 1945, **77**, 253.

[3] Astwood, E. B., *Am. J. Physiol.*, 1939, **126**, 162.

fluid and serve as the reservoir for the newly ovulated ova.[4,5] We were interested in determining whether changes in the tissue water of the oviducts paralleled those observed in the cornua and whether such changes could explain the source of the luminal fluid dilating the loops of ampullae.

Materials and Methods. Forty-one sexually mature female rats, of Wistar strain, weighing 148 to 205 g, were carefully observed to determine the onset of sexual receptivity.[6] Twenty of these females were killed at the end of heat or at the time of greatest loss of uterine tissue water.[3] The remaining 21 females were killed during proestrus or when the tissue water had reached its maximum.[3] The cornua, oviducts, and ovaries were removed intact. The utero-tubal junctions were cut and the oviducts and ovaries were put into Ringer's solution. The mesometria were carefully trimmed from the cornua and the vagina from the cervix. Each horn was then split open along its entire length, blotted on absorbent paper and weighed in weighing bottles. With the aid of iridectomy scissors and a binocular dissecting microscope, the oviducts were trimmed free of ovaries, periovarial sacs and all excess adipose tissue. A fine pipette was inserted into the fimbriated end of each oviduct and a stream of air blown through it to remove the luminal fluid. With the pipette still in place, the oviduct was removed from the Ringer's solution, brought onto absorbent paper and blotted, and the flow of air continued until it was free of fluid. The oviducts were placed in weighing dishes and weighed on a micro-analytical balance. The cornua and oviducts were subsequently dried in an oven at 110°C and reweighed. The loss of water in the cornua and oviducts was expressed in terms of per cent per 100 grams of body weight.

Results. The average percentage of water in the tissues of the cornua of females killed during proestrus was 81.8 ± 1.05 and at the end of heat 80.5 ± 0.48 (Tables I and II). The average percentage of tissue water in the oviducts in females killed during the period of proestrus was 76.5 ± 1.25 and at the end of heat 75.7 ± 0.84 (Tables I and II). There is a significant decrease in the uterine tissue water at the end of heat when compared with that of uteri removed during proestrus. The average figures presented in the tables correspond closely with those published earlier by Astwood.[3] In using the vaginal smear technic for timing his animals he reported the average per cent of uterine water during proestrus as 82.77 ± 0.35 and during late estrus as 80.37 ± 0.17.

Although there is a slight decrease (0.8%) in the tissue water of oviducts examined at the end of heat when compared with those removed during proestrus this decrease does not follow the pattern observed in the cornua. The significance of the difference between the percentage of water of the oviducts removed during proestrus as compared to those removed at the end of heat was tested by the Student's "t"-technic. The "p" value obtained ("p" = 0.027) indicates that there is no statistical significance in the differences of the two groups.

Discussion. The significant decrease in uterine tissue water between the period of proestrus and the end of heat has been confirmed. This increase in the water content of the cornua has been previously demonstrated to be due to the specific action of estrogens.[7,8] The sudden decrease in uterine tissue water during heat is thought to be due either to a decrease in estrogen production or to the action of progesterone which has an inhibitory influence on the deposition of tissue water. The origin of the fluid which dilates the ampullae of the oviducts during heat is probably not from the tissue water of this organ. The possibility of retrograde flow of uterine fluid in the normal female has been suggested but ligation of the oviducts just above the utero-tubal junction does not influence the extent to which the dilatation progresses. In addition, dyes

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[4] Huber, C. G., *J. Morph.*, 1915, **26**, 247.

[5] Odor, D. L., and Blandau, R. J., *Anat. Rec.*, 1947, **97**, 84.

[6] Blandau, R. J., Boling, J. L., and Young, W. C., *Anat. Rec.*, 1941, **79**, 453.

[7] Astwood, E. B., *Endocrin.*, 1938, **23**, 25.

[8] Van Dyke, H. B., and Ch'en, G., *Am. J. Anat.*, 1936, **58**, 473.

TABLE I.

Total Weight, Dry Weight, and Percentage Water of Uteri and Oviducts of Rats Killed at the End of Heat.

			Uteri			Oviducts		
Animal No.	Hr after onset of heat	Body wt	Total wt, mg per 100 g body wt	Dry wt, mg per 100 g body wt	% water	Total wt, mg per 100 g body wt	Dry wt, mg per 100 g body wt	% water
4	15	152	256.1	51.6	79.8	9.212	2.279	75.3
5	17	165	349.0	66.1	81.1	10.317	2.492	75.9
7	14	180	216.2	40.8	81.1	8.069	1.939	76.0
15	13	195	228.7	42.9	81.2	7.617	1.899	75.1
17	12	185	240.8	48.0	79.7	7.422	1.775	76.2
18	12	180	296.2	57.3	80.7	9.128	2.168	76.3
19	12	185	289.1	56.9	80.3	8.178	1.937	76.3
20	12	148	438.8	84.9	80.7	10.475	2.528	75.9
21	14	149	254.6	51.5	79.8	9.351	2.168	76.8
22	14	175	222.5	45.3	79.7	7.166	1.858	74.1
23	12	172	259.7	50.2	80.7	7.014	1.641	76.6
24	12	150	230.9	45.2	80.4	7.608	1.800	76.4
25	12	200	298.0	57.9	80.1	5.874	1.419	75.8
26	13	170	288.7	56.1	80.6	6.589	1.558	76.7
27	14	155	230.3	45.7	80.2	8.049	1.937	75.9
28	12	175	346.4	67.0	80.7	8.502	2.041	76.0
29	12	152	254.4	50.4	80.2	8.564	2.068	75.9
30	13	164	257.6	50.1	80.5	6.358	1.560	75.5
31	13	172	262.6	51.4	80.4	7.561	1.958	74.5
32	13	182	411.3	76.9	81.3	6.875	1.827	73.5
	Avg		281.6	54.8	80.5	7.996	1.943	75.7
	σ		59.91	11.04	0.48	1.217	0.284	0.84

TABLE II.

Total Weight, Dry Weight and Percentage Water of Uteri and Oviducts of Rats Killed During Proestrus.

			Uteri			Oviducts		
Animal No.	Hr after onset of heat	Body wt	Total wt, mg per 100 g body wt	Dry wt, mg per 100 g body wt	% water	Total wt, mg per 100 g body wt	Dry wt, mg per 100 g body wt	% water
8	88	180	300.2	51.0	83.0	8.420	2.052	75.6
9	88	170	281.6	86.0	82.0	7.829	1.966	74.9
10	89	165	361.5	62.4	82.7	9.382	2.257	76.0
11	89	205	285.9	49.6	82.7	6.756	1.721	74.5
14	84	175	268.2	45.9	82.9	7.504	1.919	74.5
16	88	174	383.7	69.4	81.9	8.190	2.040	75.1
51	84	172	284.9	50.3	82.5	8.389	1.863	77.8
52	86	174	243.3	44.3	81.8	7.926	1.803	77.3
53	86	222	319.4	57.3	82.1	5.416	1.255	76.8
54	87	184	283.2	50.5	82.2	8.401	1.830	78.4
55	88	182	346.6	69.7	79.9	8.145	2.007	75.4
56	87	166	391.0	70.5	82.0	9.645	2.183	77.4
57	87	160	325.7	62.9	80.7	8.196	1.962	76.1
58	87	176	258.6	46.0	82.2	8.118	1.773	78.2
59	88	171	209.3	40.3	80.8	6.762	1.597	76.4
60	82	186	213.1	45.0	78.4	7.446	1.853	75.2
61	85	188	354.8	65.3	81.6	7.372	1.752	76.2
62	85	178	318.1	57.5	82.0	8.651	1.981	77.1
63	83	180	212.2	39.3	81.5	9.355	2.003	78.6
64	88	182	293.1	53.6	81.7	8.033	1.834	77.2
65	88	180	280.2	49.4	82.4	8.092	1.789	77.9
	Avg		295.9	55.5	81.8	8.001	1.878	76.5
	σ		51.71	11.66	1.05	0.932	0.206	1.25

injected into the cornua during heat do not find their way into the ampullae of the oviducts. Follicular fluid may contribute to the luminal fluid but the ampullar loops have already become distended before ovulation occurs. It has been postulated that there is an increased flow of fluid from the coelomic cavity into the oviduct at the time of ovulation.[9,10] From the investigations of Alden,[11] and our unpublished data, it seems likely that the periovarial sac opening is functionally closed during the period of heat.

Summary. The water content of the uterus and oviducts of adult rats during the period of proestrus and the end of sexual receptivity has been determined. There is a significant decrease in the water content of the uterine tissue during the period of heat. No significant decrease was found in the water content of the oviducts examined during this same period.

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[9] Horst, C. J., *S. African J. M. Sc.*, 1943, **8**, 41.

[10] Westman, A., *Acta Obstet. et Gynecol. Scand.*, 1926, **5**, 1.

[11] Alden, R. H., *Anat. Rec.*, 1942, **83**, 421.

16986

Coproantibody Response in Humans Following T.A.B.* Vaccination.

R. J. Goodlow, S. C. Rittenberg, and J. H. Silliker.
(Introduced by John F. Kessel.)

From the Department of Bacteriology, University of Southern California, Los Angeles.

The value of coproantibody determination in the laboratory diagnosis of various enteric infections has been discussed recently by Harrison and Banvard.[1] The presence of agglutinins in feces was first described by Davies,[2] but this work was overlooked until the Russian investigators, Predpechensky and Moroz,[3] Skochko,[4] and Yampolsky, Gorfunkel and Aronina,[5] utilized the presence of fecal antibodies in the laboratory diagnosis of bacillary dysentery.

According to all previous investigators, agglutinins in the feces appear sooner than agglutinins in the blood serum in cases of actual enteric infection. This might suggest a local formation of the antibody in the tissue of the intestinal tract. Burrows and coworkers[6,7] indicate that antibody is excreted in the feces of animals and humans vaccinated with *V. cholerae* antigen. It is difficult to conceive how the subcutaneous injection of dead cells could stimulate the local formation of antibodies in the intestinal tract. Their appearance under these circumstances might indicate rather some mechanism of spill over from the blood stream. This view is substantiated in part by the observations of the Russian workers who found a high correlation between the presence of cellular exudate in the stool and fecal antibodies in cases of bacillary dysentery. Stools which contained large numbers of red blood cells and leucocytes commonly contained agglutinin in higher titer than stools which showed little or no cellular exudate. Burrows *et al.*,[7] however, emphasize that fecal antibodies reach their peak titer before serum antibody and tend to decline before the maximum serum

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* T.A.B.—Typhoid, Paratyphoid A and Paratyphoid B vaccine.

[1] Harrison, P. E., and Banvard, J., *Science*, 1947, **106**, 188.

[2] Davies, A., *Lancet*, 1922, **2**, 1009.

[3] Predpechensky, S., and Moroz, O., *Zh. Mikrobiol. Epidemiol.*, 1940, **7**, 3.

[4] Skochko, T. I., *Zh. Mikrobiol. Epidemiol. Immunol.*, 1942, **3**, 59.

[5] Yampolsky, S. M., Gorfunkel, D. M., and Aronina, V. B., *Pediatriya*, 1944, **5**, 13.

[6] Burrows, W., Havens, I., *J. Inf. Dis.*, 1948, **82**, 231.

[7] Burrows, W., Elliott, M. E., and Havens, I. J., *J. Inf. Dis.*, 1947, **81**, 261.

TABLE I.

Blood Serum Agglutinin Titer and Coproantibody Response in Humans Following T.A.B. Vaccination.

		Serum titer† Day				Stool titer. 0 except where indicated Day									
Subject	Organism*	0	7th	14th	24th	0	3	5	7	10	12	14	17	19	24
1	T	0	640	1280										§	
	A	0	80	640										,,	
	B	0	160	320										,,	
2	T	0	320	1280	1280					§				,,	
	A	0	160	640	1280					,,				,,	
	B	0	20	320	640					,,				,,	
3‡	T	0	0	80	320							§			
	A	0	80	640	320							,,			
	B	0	80	1280	320							,,			
4	T	20	320	320	640									8	
	A	0	80	640	640									8	
	B	0	40	80	640	5									
5‡	T	0	640	1280	160										
	A	0	160	320	320									16	
	B	0	20	160	160										
6	T	0	160	1280	1280										
	A	0	20	1280	2560										16
	B	0	80	640	2560										
7	T	0	320	640	640										
	A	0	120	320	1280										
	B	0	20	160	640										
8	T	0	160		1280										
	A	0	160		1280										
	B	0	20		640										32
9	T	20	160	80	1280		8								
	A	80	160	1280	1280									32	
	B	0	20	80	640										
10	T	40	640	640	640										
	A	0	80	640	1280										32
	B	0	40	640	640										
11	T	0	160	640	640			8							
	A	0	20	1280	640			8						32	
	B	0	0	160	640			8							
12	T	0	320	1280	2560										
	A	0	40	640	640										
	B	0	20	160	160										
13	T	40	640	1280	640										§
	A	0	80	1280	640								§	16	,,
	B	20	80	1280	1280								,,		,,
14	T	80	160	1280	1280										
	A	0	40	1280	2560										
	B	0	20	1280	2560										
15	T	40	640	1280	640								8		
	A	0	80	1280	640									64	
	B	10	80	640	1280				8						
16‡	T	0	40	320	160		§	§			16		§		
	A	0	0	160	160		,,	,,			16		,,	64	
	B	10	0	80	0		,,	,,					,,		
17	T	10	320	640	160						§				
	A	0	20	160	320						,,				
	B	0	20	320	160						,,				
18	T	160	640		1280									8	
	A	0	20		1280									64	32
	B	0	20		320									16	
19	T	80	640	1280	1280									64	
	A	0	20	1280	2560									64	
	B	0	20	640	1280										
20	T	40	640	1280	2560					32	32				
	A	10	320	1280	1280					8	64			64	
	B	0	80	1280	640					16	16				

* T—titer vs. *S. typhi;* A—titer vs. *S. paratyphi* A; B—titer vs. *S. paratyphi* B.

† Expressed as reciprocal of highest positive dilution.

‡ No history of previous T. A. B. vaccination. § No record.

titer is reached. They conclude that fecal antibody is independent of serum antibody and therefore not derived from it.

Since many of our students are routinely vaccinated with T.A.B. vaccine, it appeared to us an ideal opportunity to study the relation of vaccination and coproantibody excretion with antigens different from the cholera antigen used by Burrows.

Experimental. Twenty human volunteers were chosen for vaccination. Of this group, 7 were females and 13 males. Three of the subjects had no previous history of T.A.B. vaccination. The remainder had had no injections within the previous 2 years.

The vaccine contained 1,000 million *S. typhi* per ml, 500 million *S. paratyphi A* per ml, and 500 million *S. paratyphi B* per ml. The initial injection consisted of 0.5 ml of vaccine administered subcutaneously, followed by 1.0 ml injections, similarly administered, 7 and 14 days later.

Agglutination tests against the homologous organisms were performed using blood serum and fecal extract collected before the initial injection of vaccine, periodically throughout the series of injections and for 10 days after the final injection. The agglutination titers were determined by the usual tube technic.

The stool extracts were prepared as follows using the technic described by Harrison.[8] Morning stool specimens were collected in clean stoppered bottles the day of the test. The samples for the most part were formed or semi-formed. To each bottle approximately 2 volumes of 0.6% formalinized saline were added per volume of feces. The mixture was then homogenized in a mechanical shaker for 30 minutes and placed in the refrigerator for overnight extraction. The following morning approximately 10 ml of the mixture was placed in a centrifuge tube and spun at 3000 r.p.m. for one hour. Five ml of the relatively clear supernate was further diluted with an equal volume of formalinized saline and recentrifuged for the same period of time. In most cases this procedure gave a clear solution which was considered a 1:4 dilution of feces. In certain instances the extract did not clear completely, but this did not appear to affect materially the subsequent results. It seems to us, however, that should the determination of coproantibodies come into general use, better and more rapid methods of clarification of the stool extract must be developed perhaps by the use of settling agents. The fecal extracts were used as a source of agglutinin in the usual tube agglutination test technic. The agglutination tubes were incubated at a temperature of 45°C overnight and read with the aid of the concave surface of a microscope mirror as is done commonly in the reading of Kahn tests.

The data obtained are presented in Table I. With one exception (No. 16), all subjects developed significant blood serum agglutinin titers against all 3 organisms. In most cases the maximum titer was reached on the fourteenth day following the initial injection of vaccine. No agglutinins were detected in fecal extracts of 7 subjects at any time during the course of the experiment despite the presence of significant serum titers. Of the remaining subjects fecal agglutinins against only one organism in the vaccine mixture were found in 5 individuals, against 2 organisms in 3 subjects and against all 3 organisms in 5 subjects. Seven volunteers showed fecal agglutinins against *S. typhi,* 14 against *S. paratyphi A*, and 5 against *S. paratyphi B* at some time during the course of the experiment. The appearance of fecal agglutinins against the homologous organisms in a particular individual was extremely irregular, in 18 instances being detected only once, in 6 instances twice, and in 2 cases 3 times. No correlation is apparent between the presence of fecal antibody and the antibody titer of the blood serum; nor is there any obvious relationship between fecal antibody appearance and the time and number of antigen injections.

Summary. These results fail to indicate any rise in fecal agglutinin titer prior to the appearance of blood serum agglutinins. In this respect and also in regard to the irregularity of antibody appearance in the feces of human subjects following T. A. B. vaccination our results differ from the regular and rapid appearance of coproantibody reported to follow cholera vaccination.

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[8] Harrison, P., personal communication.

16987

Effect of Zinc on Serum Alkaline Phosphatase Activity in Patients With and Without Cancer.*

OSCAR BODANSKY AND OLGA BLUMENFELD.

From the Memorial Hospital and the Sloan-Kettering Institute for Cancer Research, New York City.

Roche and his associates[1,2] have reported that a concentration of 10^{-5} M Zn^{++} activated the serum alkaline phosphatase activity in 96 out of 101 individuals who were either normal or had non-malignant disease; the degree of activation ranged from 0 to 87%. In contrast, this concentration of Zn^{++} inhibited the serum alkaline phosphatase activity in 156 out of 185 patients with cancer; the effects in this group ranged from an activation of 24% to an inhibition of 73%.

Upon subjecting these results to statistical analysis, we found that the mean values were 25% for the degree of activation and 14% for the degree of inhibition. The difference between these two values was 39% or about 20-fold the standard error of the difference between the two means (2.1%) and hence highly significant. It was, therefore, considered of importance to determine the extent to which Roche's results were applicable to a population of patients with cancer in this hospital and, in addition, to investigate the effects of other concentrations of Zn^{++}.

The method for the determination of serum alkaline phosphatase was essentially that of A. Bodansky.[3] The pH, determined electrometrically, of the serum-substrate-buffer mixture in the absence of any added Zn^{++} was 8.75 ± 0.05. It was found, in preliminary experiments, that addition of $ZnSO_4$ to give the desired concentrations of Zn^{++} caused a shift of the pH towards the acid side. Consequently, in all determinations where Zn^{++} was present, appropriate adjustment to give a final pH of 8.75 was made by the addition of a very small volume of 0.1 or 0.75 N NaOH. The pH was checked electrometrically in all determinations of phosphatase activity reported in the present work. The effect of Zn^{++} was evaluated as the ratio of the phosphatase activity in the presence of this ion to that in its absence.

Results. Table I shows that in a group of normal individuals and in patients without malignancy, the ratios at 10^{-5}, 10^{-4}, and 10^{-3} M Zn^{++} were, respectively, 1.01, 0.89, and 0.50. When these results were evaluated statistically, it was found that only the latter two values represented significant changes (Footnote, Table I). In other words, in this group, 10^{-5} M Zn^{++} had no effect on the serum alkaline phosphatase activity, whereas 10^{-4} M Zn^{++} inhibited the phosphatase activity about 10% and 10^{-3} M inhibited it about 50%.

The effect of Zn^{++} on the activity of serum alkaline phosphatase activity was studied in a group of patients with cancer in which the following were the types chiefly represented: leukemia, Hodgkin's disease, carcinoma of the colon, cervix, uterus, stomach, bladder, and breast. Examination of the ratios at each concentration of Zn^{++} showed that there was no apparent correlation of these ratios to age, sex, type of malignancy, level of phosphatase activity, or the pre-operative or post-operative state of the patient. Accordingly, the mean value for the effect of a particular concentration of Zn^{++} was calculated for the whole group of patients. The ratios at 10^{-5}, 10^{-4}, and 10^{-3} M Zn^{++} were, respectively, 1.00, 0.90, and 0.45. Again, only the latter two

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* This investigation was supported by a research grant from the National Cancer Institute of the National Institute of Health, U. S. Public Health Service.

[1] Roche, J., van Thoai, N., Marcelet, J., and Desruisseaux, G., *C. R. Soc. de biol.*, 1946, **140**, 632.

[2] Roche, J., van Thoai, N., Marcelet, J., Desruisseaux, G., and Durand, S., *Bull. Acad. Med., Paris*, 1946, **130**, 294.

[3] Bodansky, A., *J. Biol. Chem.*, 1933, **101**, 93.

TABLE I.
Effect of Zinc Ion on Serum Alkaline Phosphatase Activity in Patients With and Without Cancer.

Group	No. of cases	Ratio of phosphatase activity in presence of zinc to that in its absence		
		Range	Mean	Stand. dev.†
Individuals without cancer*				
a. At 10^{-5} M Zn^{++}	25	0.87-1.12	1.01	0.056
b. At 10^{-4} M ”	14	0.81-1.04	0.89	0.066
c. At 10^{-3} M ”	16	0.42-0.60	0.50	0.055
Patients with cancer				
d. At 10^{-5} M Zn^{++}	39	0.94-1.07	1.00	0.025
e. At 10^{-4} M ”	22	0.77-1.00	0.90	0.058
f. At 10^{-3} M ”	24	0.27-0.60	0.45	0.079

* This group consisted of normal individuals and patients with miscellaneous diseases, chiefly heart disease. We are indebted to Dr. Harry Gold for the use of the latter patients from the Beth Israel Hospital Cardiac Clinic.

† The values for the standard deviation were used in calculating "P" values. The "P" values for the comparison of groups *a* with *d*, *b* with *e*, and *c* with *f* were, respectively, 0.4, 0.6, and 0.04. The "P" values for the comparison of groups *a* with *b*, *a* with *c*, *d* with *e*, and *d* with *f* were all less than 0.01.

values represented significant changes. Thus, in patients with cancer, as in those without, 10^{-5} M Zn^{++} had no effect on the serum alkaline phosphatase activity, whereas 10^{-4} and 10^{-3} M Zn^{++} produced significant decreases.

Statistical treatment showed that there was no significant difference between the mean values for the ratios of the individuals without cancer and the patients with cancer at any of the 3 concentrations of Zn^{++}. In view of the high "P" values for these differences, it was considered very unlikely that the extension of the study to larger numbers of patients would bring the "P" value down to 0.01 and render the differences significant.

Summary. A concentration of 10^{-5} M Zn^{++} failed to affect the serum alkaline phosphatase activity in individuals either with or without cancer. These results are not in agreement with the findings of Roche *et al.*[1,2] that this concentration of Zn^{++} activates the serum alkaline phosphatase activity of individuals without cancer and inhibits this activity in patients with cancer. Concentrations of 10^{-4} and 10^{-3} M decreased the serum alkaline phosphatase activity, but the extent of these decreases was the same in patients with and without malignancy.

16988

Multiplication of Influenza Virus in Dead Chick Embryos.

O. Lahelle and Frank L. Horsfall, Jr.
From the Hospital of The Rockefeller Institute for Medical Research, New York City.

Recently, the cultivation of *Rickettsia prowazeki* in dead chick embryos was described by Rabinowitz, *et al.*[1] These workers showed that the rickettsiae employed multiplied abundantly in embryos which, although dead, still contained surviving cells. They confirmed the earlier work of Bucciante[2] and demonstrated that dead embryos may contain living cells for surprisingly long periods. After holding 3-day-old embryos at 4°C for 24 hours, then at room temperature for 7 days, and finally at 37°C for 16 days, they were able to culture living cells from the embryo or its membrane in plasma clots.

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[1] Rabinowitz, E., Aschner, M., and Grossowicz, N., Proc. Soc. Exp. Biol. and Med., 1948, **67**, 469.

[2] Bucciante, L., *Arch. exp. Zellforsch.*, 1931, **11**, 397.

It seemed of both practical and theoretical interest to determine whether influenza virus would multiply in dead chick embryos. A number of experiments were carried out in which the time and temperature, at which embryos were held, were varied over wide ranges. The results obtained indicate that the PR8 strain of influenza A virus is capable of multiplication in the tissues of dead chick embryos if incubation at 35°C is sufficiently prolonged. They show also that the virus multiplies in dead embryos to an extent comparable to its multiplication in the living embryo.

Materials and Methods. The PR8 strain of influenza A virus was employed. It was cultivated in the allantoic sac of 9-day embryos which were incubated at 39°C before inoculation and at 35°C thereafter. Infected allantoic fluid gave hemagglutination and embryo infectivity titers of the order of 1:2000 and $10^{-8.5}$, respectively. Infected fluid was diluted in sterile nutrient broth; 0.2 cc of either a 10^{-3} or 10^{-6} dilution was employed as the inoculum.

The embryos of White Leghorn eggs were used. They were incubated at 39°C for periods ranging from 5-10 days. In certain experiments, after inoculation of the virus into the allantoic sac, embryos were held at room temperature for 3-10 days, and thereafter incubated at 35°C for 2-10 days. In other experiments, normal embryos were chilled at 4°C for either 20 or 96 hours, some of the former then held at room temperature for 3 days, after which they were inoculated into the allantoic sac with virus. Following this, the embryos were held at room temperature for an additional period of 2-10 days, and finally incubated at 35°C for 2-10 days. Groups of 2-3 embryos were employed and a variety of different experimental conditions were used. Specimens of allantoic fluid were removed repeatedly from individual embryos, usually at intervals of 2 days. In this way it was possible to follow the multiplication of the virus in individual embryos.

Measurement of the concentration of virus in the extra-embryonic fluid was carried out by the hemagglutination technique as described previously.[3] Washed chicken RBC in a final concentration of 0.25% were used. The allantoic fluid dilution-RBC mixtures were held at room temperature for one hour. The titration end point was taken as that dilution which gave a 2+ reaction. Hemagglutination-inhibition tests[3] with specific immune rabbit sera were carried out with a constant dilution of serum capable of inhibiting hemagglutination by 128 units of the homologous virus.

Virus infectivity titrations were carried out as described previously.[4] Serial tenfold dilutions of fluid were inoculated into the allantoic sac of 9-day chick embryos and, after incubation at 35°C for 48 hours, the allantoic fluids were removed and tested by the hemagglutination procedure. The infectivity end point was calculated in the usual way.

Results. It was found that the time required to kill embryos at room temperature varied somewhat with their age. Thus, 5-day embryos held at room temperature for one day were almost always dead; 8-day embryos held for 2 days were usually dead, and invariably when held for 4 days; 10-day embryos held for 3 days were usually dead, and invariably when held for 4 days. Only 5-day embryos were held at 4°C and in every instance it was found that 20 hours at this temperature resulted in death. Embryos were considered to be dead when spontaneous movements had ceased, blood vessels showed no pulsation and no further development occurred.

The results obtained with embryos held for long periods at room temperature after inoculation with the PR8 strain are shown in Table I. It was found that multiplication of the virus did not occur when embryos were kept at room temperature for periods up to 8 days and not incubated at 35°C thereafter. However, with inoculated embryos which had been held at room temperature for 7-10 days, and then incubated at 35°C for periods ranging from 2-10 days, definite evidence of mul-

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[3] Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

[4] Hirst, G. K., *J. Immunol.*, 1942, **45**, 285.

TABLE I.
Multiplication of PR8 in the Allantoic Sac of Chick Embryos Held at Room Temperature for Long Periods After Inoculation.

		After inoculation			Allantoic fluid	
Age of embryos (days)	Inoculum 0.2 cc intra-allantoic (dilution)	Room temp. 22-25°C (days)	35°C (days)	No. embryos	Hemagglut. titer*	Embryo-infectivity titer
5	10^{-3}	3-8	—	3	0,0,0	
7	”	”	—	”	0,0,0	
9	”	”	—	”	0,0,0	
5	”	7	2	2	8 16	
”	”	”	4	”	128,128	
”	”	”	8	”	256,256	$>10^{-5}$
8	”	”	2	”	0,0	
”	”	”	4	”	0,128	
”	”	”	8	”	512,2048	$>10^{-5}$
10	”	”	2	”	0.0	
”	”	”	4	”	0.0	
”	”	”	8	”	0 32	
5	”	10	3	”	0 32	
”	”	”	5	”	8,256	
”	”	”	7	”	512,2048	$>10^{-5}$
8	”	”	3	”	0 0	
”	”	”	5	”	0.32	
”	”	”	7	”	C,128	$>10^{-5}$
5	10^{-6}	7	2	3	0,0,128	
”	”	”	6	”	C,1024,1024	
”	”	”	10	”	C,2048,2048	
5	”	10	3-10	”	0,0.0	

* Hemagglutination titer of allantoic fluid from individual embryos.
C = Bacterial contamination.

tiplication of the virus was obtained. In contrast to what occurs in the allantoic sac of living embryos,[5] maximum hemagglutination titers were not obtained with dead embryos until the period of incubation at 35°C had been extended to 6 or 8 days. In certain instances hemagglutination titers as high as 1:2000 and embryo infectivity titers of at least 10^{-5} were obtained with fluid from dead embryos. Hemagglutination-inhibition tests with immune sera served to identify the virus; complete inhibition was demonstrated repeatedly with anti-PR8 serum, while anti-Lee serum did not inhibit the reaction. Even when the inoculum was as small as 10^{-6}, *i.e.* approximately 10^2 E.I.D., multiplication of the virus occurred. It is noteworthy that inoculated embryos which were held for 10 days at room temperature were capable of supporting the multiplication of influenza virus when they were incubated at 35°C. It should be reiterated that, irrespective of the age of the embryos employed, none was capable of surviving for longer than 4 days at room temperature.

The results obtained with embryos which were killed by chilling at 4°C, and held for various periods at room temperature before inoculation with the PR8 strain, are shown in Table II. In these experiments all embryos employed were 5 days of age. In the large majority of instances embryos which had been inoculated after death were capable of supporting the multiplication of influenza virus if they were incubated at 35°C. Moreover, dead embryos held at room temperature for 3 days before inoculation or 4 days there-

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[5] Pearson, H. E., *J. Bact.*, 1944, **48**, 369.

TABLE II.
Multiplication of PR8 in the Allantoic Sac of Chick Embryos Held at 4°C or at Room Temperature for Long Periods Before Inoculation.

Before inoculation			After inoculation			
4°C (hr)	Room temp. 22-25°C (days)	Inoculum 0.2 cc intra-allantoic (dilution)	Room temp. 22-25°C (days)	35°C (days)	No. embryos*	Allantoic fluid hemagglut. titer†
20	—	10^{-3}	4	2	3	64,64,64
"	—	"	"	6	"	512,256,256
"	—	"	"	10	"	128,128,64
"	—	"	9	2-6	"	0,0,0
"	—	10^{-6}	4	2	"	0,0.0
"	—	"	"	4	"	C,2048,64
"	—	"	"	10	"	C,2048,2048
"	—	"	7	3-9	"	C,0,C
"	—	"	10	2-5	"	C,0,C
—	4	10^{-3}	4	3	"	256,0,64
—	"	"	"	6	"	32,1024,128
—	"	"	8	3	2	64,0
—	"	"	"	7	"	16,C
20	3	"	4	3	3	64.0,0
"	"	"	"	6	"	512,C,0
"	"	"	"	10	"	512,C,C
"	"	"	8	3-7	"	0,0,0
96	—	"	—	2	"	0,0,8
"	—	"	—	5	"	512,512,1024
"	—	"	—	11	"	2048,2048,2048
"	—	"	2	3-9	"	0,0,0
"	—	"	4	3-10	"	0,C,C
"	—	"	8	3-7	"	0,0,0

* All embryos were 5 days of age when inoculated.
† Hemagglutination titer of allantoic fluid from individual embryos.
C = Bacterial contamination.

after supported viral multiplication. Maximum hemagglutination titers comparable to those obtained with live embryos were demonstrated with dead embryos when incubation at 35°C was carried out for 4-6 days. Quantities as small as 10^2 E.I.D. of virus were capable of initiating multiplication in such embryos. It is of interest that even when embryos were held at 4°C for as long as 96 hours, they were still capable of supporting multiplication of the virus. It should be emphasized that such embryos had been dead for at least 3 days before inoculation.

In other experiments embryos were frozen at –30°C and held for 20 hours. They were then thawed, inoculated with a 10^{-3} dilution of virus and incubated at 35°C for prolonged periods. Under these conditions no evidence of multiplication was obtained in any instance.

The extra-embryonic fluid obtained from dead embryos had a gross appearance very different from that which is withdrawn from live embryos. In most instances the fluid was cloudy or turbid and frequently it contained some yolk, albumin, or both. As might be expected, hemagglutination reactions obtained with such fluids were somewhat abnormal, especially in the lower dilutions. However, in the higher dilutions hemagglutination appeared to be nearly normal and the end point was usually determined easily.

Discussion. Multiplication of influenza virus in the tissues of dead chick embryos is probably analogous to its multiplication in chick embryo tissue culture medium. It was surprising, however, to find that when dead embryos were incubated at 35°C for a sufficient period the virus titer became much higher than is found in liquid tissue culture.[6] The evidence obtained does not suggest that the virus is capable of multiplying in the absence of living cells. It has been demonstrated clearly[1,2] that in embryos killed by chilling at 4°C or by prolonged storage at room temperature, living cells are present. Presumably these cells carry on their usual metabolic functions when held at 35°C. It seems probable that the long period of incubation necessary for the development of maximal virus titers may be related to and dependent upon the multiplication of such living cells in both the dead embryo and its membranes. In this connection it will be recalled that when embryos were frozen at –30°C they were thereafter incapable of supporting viral multiplication.

It is apparent that these results may be of practical usefulness, especially in field work where attempts are made to recover viruses from infected persons or animals. In the case of influenza virus, at least, it seems evident that it is not necessary to observe special precautions with chick embryos. When adequate laboratory facilities are lacking, dead embryos which have been stored for considerable periods may prove satisfactory for the recovery of the infectious agent.

Summary. Chick embryos killed by prolonged storage at room temperature or 4°C are capable of supporting the multiplication of influenza virus upon prolonged incubation at 35°C.

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[6] Weller, T. H., and Enders, J. F., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 124.

16989 P

Relation of Vitamin B_{12} to Liver Basophilia.*

JOEL R. STERN, M. WIGHT TAYLOR, AND WALTER C. RUSSELL.

From the Department of Agricultural Biochemistry, New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, N.J.

Vitamin B_{12} has been reported to stimulate the growth of chicks on a vegetable protein diet and of certain lactobacilli.[1,2] When it was later found that the nucleoside thymidine can replace vitamin B_{12} in the medium of these microorganisms, it was postulated that the vitamin acts as an enzyme in the synthesis of nucleosides and thus of nucleotides and nucleic acids.[3,4] It was decided to test this theory by studying the formation of nucleic acids in the rat as influenced by vitamin B_{12}. Since it is generally accepted by histochemists that the basophilia reaction determines ribonucleoprotein,[5] the effect of vitamin B_{12} on the basophilia of rat livers was studied in this experiment.

Experimental. Weanling white rats whose mothers had been fed a diet in which the sole source of protein was soybean oil meal were used in this investigation. When the young were 21 days old they were removed from the mothers and divided into 3 groups. Groups 1 and 2 received a diet of the purified type in which soybean oil meal was the sole source

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* Journal Series paper of the New Jersey Agricultural Experiment Station, Rutgers University, the State University of New Jersey, Department of Agricultural Biochemistry.

[1] Ott, W. H., Rickes, E. L., and Wood, T. R., *J. B. C.*, 1948, **174**, 1047.

[2] Shorb, Mary L., *Science*, 1948, **107**, 397.

[3] Wright, L. D., Skeggs, Helen R., and Huff, J. W., *J. B. C.*, 1948, **175**, 475.

[4] Shive, W., Ravel, Joanne M., and Eakin, R. E., *J. Am. Chem. Soc.*, 1948, **70**, 2614.

[5] Demsey, E. W., and Wislocki, G. B., *Phys. Rev.*, 1948, **26**, 1.

TABLE I.
Relation Between Diet and Basophilia.

Group	Rat No.	Dietary supplement	Gain, g per day	Basophilia, grade
1	1	None, negative control	0.8	0
	2	,, ,, ,,	1.2	0
	4	,, ,, ,,	1.0	+
	5	,, ,, ,,	2.5	0
	22	,, ,, ,,	0.9	+
	28	,, ,, ,,	1.3	0
	29	,, ,, ,,	0.6	0
	36	,, ,, ,,	1.0	0
2	3	B_{12}	4.2	+++
	6	,,	2.4	+++
	24	,,	4.3	+++
	39	,,	4.4	++
3	27	Liver	5.1	++++
	34	,,	4.0	++++

of protein. Vitamin B_{12}[†] was included in the diet of Group 2 at a level of 440 μg per kilo. Group 1, the diet of which was not supplemented, served as the negative control. For Group 3, the diet was modified by the introduction of 4.2 g of dried liver containing 2.5% protein per 100 g of diet at the expense of the soybean oil meal. All diets contained 22% protein and included vitamins and salt mixture in optimal amounts. The pups were allowed to feed and drink *ad libitum* and were sacrificed at the end of the 21-day experimental period.

Segments of liver were removed immediately after the animals were sacrificed, fixed in Carnoy's fluid, dehydrated through alcohol, and sectioned at 8 μ. The sections were stained with eosin and methylene blue.

Results. An arbitrary scale was decided upon to evaluate the extent of the basophilic staining. Livers which showed no cytoplasmic basophilia were graded 0, and those which exhibited minimal basophilia were graded +. Moderate concentrations were graded ++; sections with more intensive staining were graded +++; and those sections whose cytoplasm was replete with basophilic staining were graded ++++.

Table I shows that rats which received vitamin B_{12} or dried liver grew at a markedly greater rate than those which received no supplement. Furthermore, the animals which gained slowly were characterized by little or no hepatic basophilia, whereas those which gained rapidly showed extensive cytoplasmic basophilia in the liver cells.

The review by Demsey and Wislocki[5] points out that basophilia, shown to be ribonucleoprotein, is intimately related to protein synthesis. It would thus seem that when animals are deprived of vitamin B_{12} the formation of nucleoprotein (through the nucleoside) is limited, and consequently protein synthesis in the body would be retarded.

Summary. The growth of weanling white rats was stimulated by inclusion of vitamin B_{12} or of whole dried liver in the diet. Rats which received no B_{12} or liver grew poorly and showed little or no liver basophilia, whereas those which received B_{12} or liver grew well and showed considerable cytoplasmic basophilia in their liver cells.

[†] Vitamin B_{12} concentrate was kindly supplied by Merck & Company, Rahway, N. J.

16990

Hypothromboplastinemia Following Total Body Irradiation.

William D. Holden, Jack W. Cole, A. F. Portmann, and J. P. Storaasli.
(Introduced by R. W. Heinle.)
From the Departments of Surgery and Radiology, Western Reserve University, Cleveland, O.

Allen and Jacobson[1] published a report concerning the hemorrhagic syndrome associated with total body exposure to ionizing irradiation. They were able to demonstrate a circulating anticoagulant which was indistinguishable from heparin. A more detailed study of the same and similar experiments was published by Allen, Sanderson, Milham, Kirschon, and Jacobson.[2] These studies are of considerable importance with respect to the effect of irradiation upon the human being and also because of their relation to idiopathic thrombocytopenic purpura and thrombocytopenia secondary to aplasia or destruction of the bone marrow. Interest has been stimulated among radiologists, investigators working in the field of atomic irradiation, hematologists, and various others concerned with the coagulation of blood.

The syndrome that Allen and his associates produced in the dog with a total body exposure of 450 r (LD-100%) consisted principally of extensive hemorrhage throughout all organs of the body, neutropenia, thrombocytopenia, anemia, prolongation of the clotting time of whole blood, and prolongation of the bleeding and clot retraction times. Prothrombin times were within normal limits up to 24 hours prior to death. Blood fibrinogen, calcium, phosphorus, and magnesium levels were normal. The animals succumbed on an average of 11 days following irradiation. This syndrome resembles that of idiopathic thrombocytopenia with the exception of the neutropenia and prolonged clotting time. The decreased coagulability of the blood was thought to be due to a circulating heparin-like anticoagulant. This was demonstrated by prolonging the clotting time of normal plasma by the addition of plasma from an irradiated animal and by *in vivo* and *in vitro* titrations of the irradiated blood with toluidine blue and protamine sulphate.

Kohn and Robinett[3] reported no change in the clotting time of rats subjected to 1,000-1,250 r, which was 125-150% of the LD-50%. By administering heparin to the rats, the LD-50% could be reduced about one-third. Allen and his associates routinely used the LD-100%.

Our interest in this problem arose in an attempt to determine whether the reputed decreased coagulability of the blood following irradiation of the dog was due to circulating heparin or an increase of the heparin cofactor. Our experiments have demonstrated no evidence of heparinemia or increase in the heparin cofactor. A definite change in the amount of thromboplastic protein that could be extracted from plasma subjected to high-speed centrifugation was found.

Method. Studies were carried out prior to irradiation and for the most part, every other day after irradiation. Determinations consisted of the hemoglobin, erythrocyte count, leucocyte count, platelet count (blood smears, 2.5% sodium citrate), clotting time,[4] clot retraction, heparin cofactor,[5] protamine titration where indicated, and thromboplastin assay.

Healthy mongrel dogs were subjected to total body irradiation with 450 r (LD-100%) from a 220 K.V.P. machine at 15 milliamperes through a 0.5 mm copper filter and a 1.0 mm aluminum filter. H.V.L. was 1.2 mm

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[1] Allen, J. G., and Jacobson, L. O., *Science*, 1947, **105**, 388.

[2] Allen, J. G., Sanderson, M., Milham, M., Kirschon, A., and Jacobson, L. O., *J. Exp. Med.*, 1948, **87**, 71.

[3] Kohn, H. I., and Robinett, P., *Oak Ridge National Laboratory*, 1948, unclassified.

[4] Lee, R. I., and White, P. D., *Am. J. Med. Sci.*, 1913, **145**, 495.

[5] Holden, W. D., Cole, J. W., and Davis, J. H., Jr., *Surg., Gyn., and Obs.*, 1949, in press.

TABLE I.
Hematologic and Coagulation Changes in Exp. No. 5.

Date	11/22	11/24	11/26	11/29	12/1	12/3	12/6	12/8
Hb. g/100 ml	16.0	Irradiated 450 r.	15.5	15.0	13.5	13.0	11.0	9.0
RBC/c.mm. × 10^6	6.56		6.23	6.03	6.12	5.59	4.02	3.62
WBC/c.mm.	15,275		10,700	2,000	600	760	175	50
Platelets/c.mm. × 10^3	308		236	223	110	16	4	
Clotting time, min.	7		5	5	9	9	9	9
Heparin cofactor. % thrombin destroyed	92.2		95	93.5	92	92.3	94.9	85
Clotting time supernatant plasma (sec.)	138		190	283	403	348	510	600
Clotting time (sec.) thromboplastin + supernatant plasma	24		21	22	29	22		
Clotting time (sec.) suspended sediment and supernatant plasma	84		89	87	120	120	132	
Clot retraction	Normal							None

of copper. Irradiation was delivered at a rate of 6-7 r per minute.

Chargaff and West[6] demonstrated that a sediment exhibiting thromboplastic activity could be removed from oxalated plasma subjected to high-speed centrifugation. The clotting time of the supernatant plasma was an index of the amount of thromboplastic protein remaining after centrifugation. The clotting time of the supernatant plasma after portions of the sediment were added to it also indicated the amount of thromboplastic activity in the original plasma. In this way, minute changes in the thromboplastic protein, measured in gammas, could be demonstrated.

The following procedure was used to assay the thromboplastic protein of the plasma. Twenty ml of blood were removed from the jugular vein with as little tissue trauma as possible and quickly mixed with 2.0 ml of a 0.1 M sodium oxalate solution. The blood was centrifuged at 4,000 r.p.m. (2,400 g) for 20 minutes at a temperature of 4°C in a refrigerated International centrifuge. The supernatant plasma was carefully pipetted off, and this was centrifuged for 150 minutes at 20,000 r.p.m. (33,000 g) at the same temperature. The plasma was decanted. The sediment remaining in the base of the centrifuge tube was then suspended in normal saline. The amount of saline used was one-tenth the volume of supernatant plasma. To 0.1 ml of the supernatant plasma was added 0.1 ml of normal saline, and the mixture was placed in a water bath at 37°C for one minute. One-tenth milliliter of 0.022 M calcium chloride solution was added and a stopwatch started. The clotting time was determined in seconds. When short clotting times were obtained, solid clot formation occurred and this was used as the end point. When the clotting time was prolonged, the formation of a solid clot was preceded by fibrin strands. End points were indicated by the first appearance of the latter. One-tenth milliliter samples of the supernatant plasma were then added to saline and various concentrations of a suspension of thromboplastin* and the clotting times determined after incubation for one minute at 37°C and recalcification. The suspension of thromboplastin was prepared by mixing 50 mg of the thromboplastin with 12 ml of normal saline and heating it for 10 minutes at 50°C. Following the above procedure, 0.1 ml of the suspended sediment was added to 0.1 ml of the supernatant plasma. The mixture was incubated for one minute at 37°C and then quickly recalcified by adding 0.1 ml of the 0.022 M calcium chloride solution.

Results. Five dogs were irradiated. One lived 5 and another 7 days, and neither showed any changes in the clotting mech-

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[6] Chargaff, E., and West, R., *J. Biol. Chem.*, 1946, **166**, 189.

* The Maltine Company.

TABLE II.
Average of Blood Determinations on the Day Before Irradiation and the Day of Death.

	Before irradiation	Day of death
Hb. g/100 ml	13.4	10.1
RBC/c.mm. × 10^6	6.5	3.8
WBC/c.mm.	12,300	175
Platelets/c.mm.	236,000	6,630
Clotting time (min.)	8	8
Clot retraction	Normal	0
Heparin cofactor. % thrombin destroyed	89	83
Clotting time (sec.) supernatant plasma	147	440
Prothrombin time (sec.)	14.8	17.4
Clotting time (sec.) supernatant plasma + sediment	70	107

anism or hematologic picture that could be subjected to a critical analysis. The other 3 dogs survived 9, 10, and 11 days respectively and displayed the same clinical changes and similar alterations in the studies of their blood. At autopsy the following findings were observed. In general, multiple focal pulmonary and epicardial hemorrhages were present. Submucosal hemorrhages were scattered throughout the gastrointestinal tract, and were routinely present in the urinary bladder. Small areas of bronchopneumonia were present in one dog, while in another, pneumonic consolidation was widespread. A hemothorax (50 ml) was present in one dog and a bilateral pleural effusion in another.

Table I shows the studies performed during the course of one experiment, while Table II shows the average determinations of 3 experiments before irradiation and on the day of death. The significant findings so far as the hemorrhagic tendency is concerned are the reduction in thrombocytes, the increase in the clotting time of the supernatant plasma, and the increase in the clotting time of the supernatant plasma after the addition of the suspended sediment (Fig. 1). These latter 2 determinations represent thromboplastic protein in the blood stream and indicate a definite diminution following irradiation. The gradual reduction of thromboplastic activity in the plasma parallels the reduction in thrombocytes, but it cannot be stated from these observations that the thromboplastic protein was derived from the thrombocytes. The following determinations demonstrate that the prolongation of the clotting time was not due to the presence of an anticoagulant or the depletion of either prothrombin or fibrinogen.

Protamine sulphate† in amounts from 0.02 mg to 0.06 mg in 0.1 ml of physiological saline was added to 0.1 ml of the supernatant plasmas after their clotting times had become prolonged. After adding 0.1 ml of 0.022 M calcium chloride solution, there was in no instance a shortening of the control clotting time. In other experiments[7] plasma obtained from heparinized blood showed a return of the prolonged clotting time to normal or near normal values after the addition of protamine sulphate. The above titrations suggest that no anticoagulant subject to neutralization by protamine was effecting the deceleration of clot formation in the supernatant plasma.

When 0.1 ml of commercial thromboplastin and 0.1 ml of 0.022 M calcium chloride solution were added to the supernatant plasma, a satisfactory estimate of the available prothrombin was determined. Clotting times varied from an average of 14.7 seconds before irradiation to 17.4 seconds on the day of death. Although this represents a slight reduction in the amount of prothrombin in the supernatant plasma, it hardly accounts for either the marked prolongation of the clotting time of the recalcified supernatant plasma or the hemorrhagic manifestations exhibited by the animals.

The fact that a tenacious solid clot could always be obtained from the recalcification of the supernatant plasma indicated that an adequate quantity of fibrinogen was present. This was further demonstrated by adding 10

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† The Upjohn Company.

[7] Portmann, A. F., and Holden, W. D., to be published.

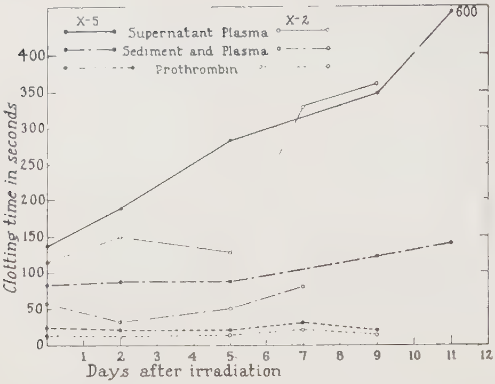

FIG. 1.

Graphic representation of the changes in supernatant plasma, sediment and plasma, and prothrombin in two experiments.

units of thrombin‡ to 0.1 ml of the supernatant plasma and 0.1 ml of 0.9% sodium chloride solution and obtaining satisfactory clots in less than 5 seconds.

Clotting times performed on whole blood throughout all experiments showed no significant changes. Determinations of the heparin cofactor likewise showed no change of importance. The failure to demonstrate any prolongation of the clotting time of the blood is at variance with the observations of Allen and his associates.[1,2] We have no satisfactory explanation of this. The determination of the clotting time of whole blood by any of the various test tube methods is notoriously difficult to perform and interpret. For this reason special precautions were taken. The same physician drew all the blood and performed all the clotting times under the same circumstances. We do not wish to deny that heparinemia may exist after irradiation of this character, but up to the present we have been unable to satisfy ourselves that it exists, whereas we have been able to demonstrate a hypothromboplastinemia and thrombocytopenia which explain the clinical manifestations exhibited by these animals.

Conclusions. 1. A hemorrhagic syndrome in dogs was produced by total body irradiation (450 r). Manifestations consisted of multiple areas of hemorrhage in the pulmonary, gastrointestinal, and urinary systems, anemia, leucopenia, thrombocytopenia, prolongation of clot retraction, and hypothromboplastinemia.

We wish to thank Mrs. Ruth Stavitsky for her technical assistance.

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‡ Parke, Davis and Company.

16991

Fluorescence of Intravenously Administered Rose Bengal Appears Only in Hepatic Polygonal Cells.

ALBERT I. MENDELOFF.* (Introduced by Robert W. Wilkins.)

From the Evans Memorial, Massachusetts Memorial Hospitals, and the Department of Medicine, Boston University School of Medicine, Boston.

Although certain intravenously administered dyes have been employed in the study of hepatic function over the past 3 decades, the exact mechanisms by which they are removed from the blood are poorly understood. Earlier work indicated that the entire reticuloendothelial system participated in the removal of these substances. This concept is still widely prevalent, although more recently indirect evidence suggests that the normal liver alone is responsible for the uptake of such dyes as the disulfonate of phenoltetrabromphthalein (Bromsulfalein).[1] Now that hepatic blood flow in man is being estimated by a technique based on the extraction of Bromsulfalein by the liver,[2] it is essential to determine whether the removal of this dye, or of others similarly handled, measures the activity of hepatic polygonal cells, or of Kupffer cells.

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* Postdoctorate Research Fellow of the National Institutes of Health.

[1] Ingelfinger, F. J., *Bull. New Eng. Med. Cen.*, 1947, **9**, 25.

In order to study this problem, advantage has been taken of the fact that tetraiodotetrachlorfluorescein (Rose Bengal), a dye introduced for the study of liver function by Kerr *et al.*[3] exhibits a soft reddish-orange fluorescence in the long ultraviolet spectrum (3150-3650 Å). Tissues stained with 1-2% aqueous solutions of this dye emitted the same characteristic fluorescence, which was readily observed through a yellow eyepiece filter on an ordinary microscope by ultraviolet light (Source—General Electric H-4 medium pressure mercury vapor lamp) passed through glass condensers, Woods filter, 5% copper sulfate solution, glass substage condenser, and glass slides. The fluorescence was not intensified when observed in an all-quartz optical system, and remained unaffected by several hours' exposure to the illumination described above.

Aqueous solutions (1-2%) were prepared from standard bacteriological Rose Bengal powder;[†] these were autoclaved, and doses varying from 20 to 100 mg were injected into the ear veins of adult rabbits lightly anesthetized with ether. Liver and splenic biopsies in 20 such rabbits were obtained from 1 to 15 minutes after injection. The tissues were immediately placed in saline, then cut on the freezing microtome into sections 15 μ thick. These were floated on water, mounted on ordinary glass slides, and examined within 10 minutes after mounting. The characteristic sustained fluorescence due to Rose Bengal was noted constantly in the hepatic polygonal cells. In biopsies made within the first 3-4 minutes following injection of the dye, the polygonal cell fluorescence was somewhat less intense than in those biopsies made at 10 minutes after injection. Along the endothelial lining of the sinusoids, where Kupffer cells are normally found, no such fluorescence was observed. One constantly saw, however, in these locations as well as in the periphery of some polygonal cells, the greenish-white, rapidly fading fluorescence ascribed by Popper and Greenberg[4] to Vitamin A. Grossly, in ordinary white light, the livers were definitely reddish. In 16 rabbits, the color of the spleen differed in no way from that of uninjected controls, but in 4 rabbits it was slightly pink. Such faint orange fluorescence as was seen in splenic sections did not appear to be located in the parenchyma.

Two anesthetized human subjects undergoing laparotomy were subjected to liver biopsy just prior to and 10 minutes following intravenous injection of 150 mg of Rose Bengal (2% solution). These biopsy tissues were prepared exactly as described above. They showed the same picture as did the rabbit livers—the orange fluorescence due to Rose Bengal in the hepatic polygonal cells, but nowhere else.

These observations provide evidence that the dye Rose Bengal is taken up by, and stored in the hepatic polygonal cells rather than in the Kupffer cells of the liver. Whether the Kupffer cells participate in the removal of the dye from the blood, although unlikely, cannot with certainty be excluded by the technique of fluorescence microscopy. All one can say is that, during a period of 1 to 15 minutes after injection of the dye, the fluorescence due to Rose Bengal is not observed in those areas where Kupffer cells are commonly found. In this respect these findings are similar to those of Grafflin[5] on the handling of fluorescein by the frog liver. Other evidence obtained in this laboratory by hepatic venous catheterization of normal man[6] shows clearly that the hepatic uptake of Rose Bengal is slowed or blocked by simultaneously-administered Bromsulfalein. It is suggested, therefore, that the removal of both these dyes is normally a function of the hepatic parenchymal cells rather than of the Kupffer cells.

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[3] Kerr, W. J., Delprat, G. D., Epstein, N. N., and Dunievitz, M., *J. A. M. A.*, 1925, **85**, 942.

[†] National Aniline Company.

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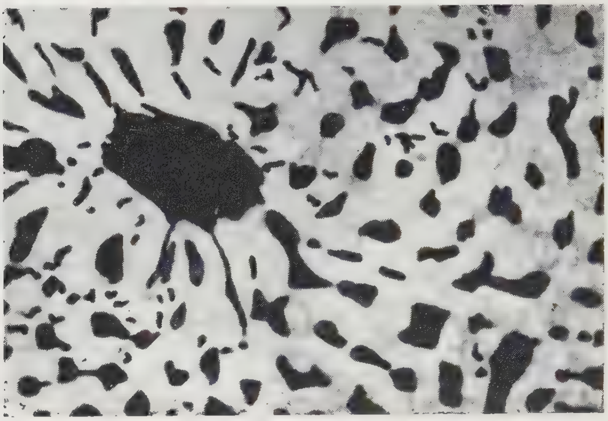

FIG. 1.
Low-power photomicrograph of rabbit liver (300 ×, enlarged 2 ×), fixed in formalin and examined unstained by ultraviolet light. The print has been retouched to emphasize the contrast between non-fluorescent sinusoids and fluorescent hepatic trabeculae, and to remove artifacts which displayed light yellow or green fluorescence.

Summary. Intravenously-administered Rose Bengal can be visualized by fluorescence microscopy in the livers of rabbits and humans. The fluorescence is seen in hepatic polygonal cells, but not in Kupffer cells. It is suggested that the polygonal cells of the normal liver are responsible for the storage of Rose Bengal, and, by analogy, Bromsulfalein.

The author acknowledges with thanks the advice and criticism of Doctors F. J. Ingelfinger, F. Sjostrand, and J. Scott.

16992

Effect of *in vivo* Addition of Selected Nitrofurans on Acid Production in Human Saliva.*

SAMUEL DREIZEN, HENRY I. GREENE, AND TOM D. SPIES.
From the Department of Nutrition and Metabolism, Northwestern University.

Investigation of the relation between nutritional status and dental caries has been a major study at the Nutrition Clinic of the Hillman Hospital, Birmingham, Alabama, since 1941. During the past year an extensive study was made of the effect of the *in vitro* addition of selected nitrofuran compounds on acid production in human saliva, and on the growth and acid production of a pure culture of a strain of the Lactobacillus acidophilus recovered from a carious lesion.[1] The antibacterial action of the nitrofurans has been reported by a number of investigators.[2-4] Our findings showed that 5-nitro-

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* Northwestern University Studies in Nutrition at the Hillman Hospital, Birmingham, Ala.

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We wish to thank Mrs. Thelma S. Brummal for her aid in making the caries activity determinations.

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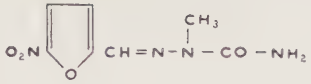
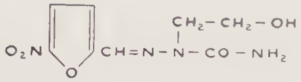
[3] Dodd, M. C., and Stillman, W. B., *J. Pharm. and Exp. Therap.*, 1944, **82**, 11.



5-nitro-2-furaldehyde-2-β-methyl semicarbazone



5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone

Fig. 1.
Structural formulae of 5-nitro-2-furaldehyde-2-β-hydroxethyl semicarbazone and 5-nitro-2-furaldehyde-2-β-methyl semicarbazone.

2-furaldehyde-2-β-hydroxyethyl semicarbazone and 5-nitro-2-furaldehyde-2-β-methyl-semicarbazone (Fig. 1), in 500 μg quantities, completely inhibited acid production in 10-cc aliquots of paraffin stimulated saliva in 90.5 and 85% of the samples respectively. A significant but incomplete inhibitory effect was observed in all other instances. Both compounds proved to be bactericidal against an oral strain of the Lactobacillus acidophilus, when added in amounts of 3.5 mg and above to yeast extract dextrose broth inoculated with the test organism.

To investigate the possibility that conditions in the mouth not reproducible *in vitro,* such as the absorption potential of the oral mucous membranes, tissue enzymes and the migration of phagocytes to the oral cavity, may interfere with the antibacterial action of 5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone and 5-nitro-2-furaldehyde-2-β-methyl semicarbazone on the organisms associated with dental caries, it was decided to determine whether these compounds retained their effectiveness as inhibitors of acid production when added *in vivo* to the saliva of persons highly susceptible to dental decay.

Materials and Methods. Sixty-nine patients, each of whom had clinical and laboratory evidence of active dental caries, were selected for this study. The clinical examination was made with a mouth mirror and exploring tine and was supplemented in most instances by bite wing or full mouth roentgenograms. Laboratory evidence of prevailing dental caries activity was established by analyzing samples of saliva by the methods of Hadley,[5] Fosdick, Hansen, and Epple,[6] and Dreizen, Mann, Cline and Spies.[7]

Method of Study. Two samples of stimulated saliva comprising 20 cc and 15 to 20 cc respectively were collected in sterile flasks from each patient. An untreated paraffin block served as the salivary stimulant for the first sample and a paraffin block containing 750 μg or 1,000 μg of the test compounds served as the secretory stimulant for the second. The collection time for each sample ranged from 10 to 20 minutes with a 15 minute interval between samples. Paraffin blocks containing 750 μg of 5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone, 1,000 μg of 5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone, 750 μg of 5-nitro-2-furaldehyde-2-β-methyl semicarbazone, and 1,000 μg of 5-nitro-2-furaldehyde-2-β-methyl semicarbazone were used for 20, 23, 7 and 19 patients respectively.

A 10-cc aliquot of the saliva sample obtained with the untreated paraffin block was tested immediately for soluble calcium by the method of Fosdick, Hansen, and Epple.[6] Ten-cc aliquots of the samples obtained with the nitrofuran-treated and untreated paraffin blocks were added to sterile brown vials containing dextrose and tricalcium phosphate. The vials were sealed, shaken, incubated and agitated in a water bath maintained at 37°C. Following a 4-hour period of incubation and agitation, the amount of soluble calcium contained in each of these aliquots was determined. The difference in calcium values between the incubated and unincubated aliquots of saliva for each patient was regarded as a measure of the degree of acid production.

Observations. The effect of the *in vivo* addition of 5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone on acid formation in the

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[4] Main, R. J., *J. Am. Pharm. Assn.*, 1947, **36**, 317.

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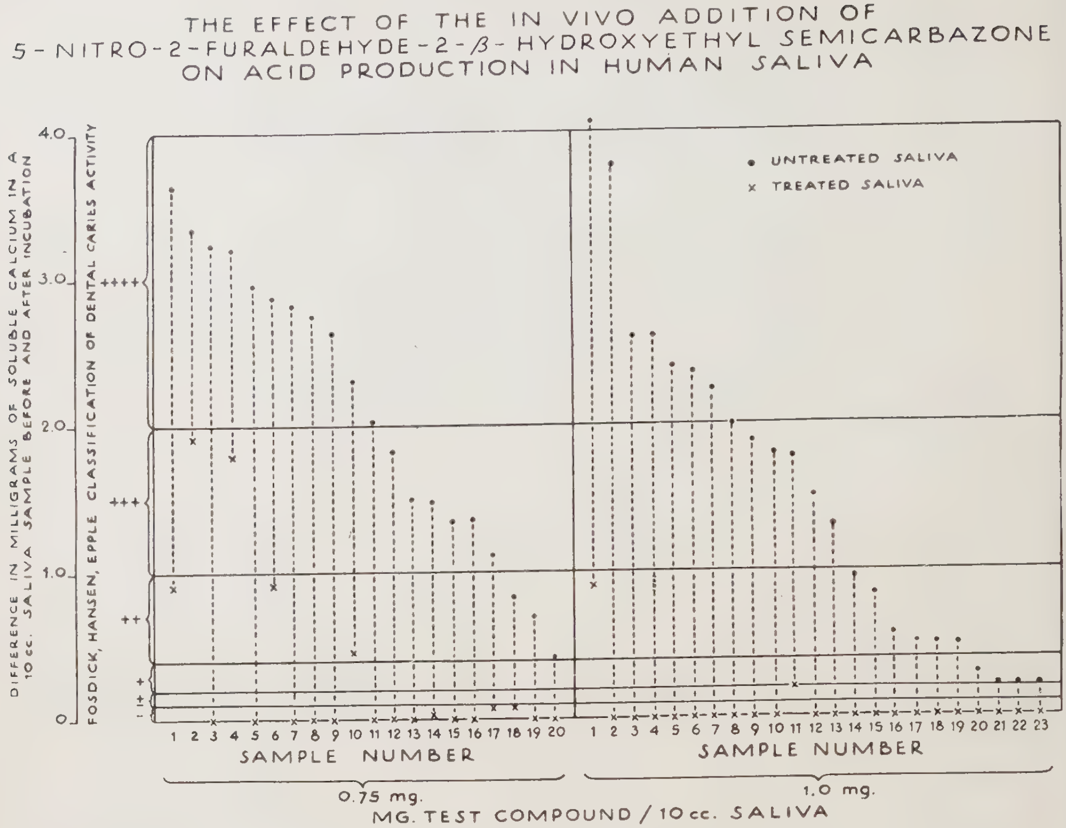

Fig. 2.

The sample numbers are arranged in the order of decreasing dental caries activity in the control aliquot and not in the order in which the samples were collected.

saliva of caries active individuals is shown in Fig. 2. When the compound was added in 750 μg amounts, acid production was completely inhibited in 15 (75%) of the 20 samples tested. A significant partial reduction in acid formation was observed in each of the remaining 5 samples. The *in vivo* addition of 1,000 μg amounts of this compound resulted in a complete inhibition of acid formation in 21 (91%) of the 23 samples tested. Acid production was reduced to a significant extent in the remaining 2 samples.

Fig. 3 demonstrates the results of the addition *in vivo* of 5-nitro-2-furaldehyde-2-β-methyl semicarbazone on acid production in the saliva of caries susceptible individuals. In the 7 instances in which 750 μg quantities of this compound were added, acid formation was completely prevented in only 1 (14%) of the samples, the amount of acid formed in the untreated aliquot of this sample being negligible. Acid production was deterred, however, to a significant degree in each of the remaining samples. When the amount of 5-nitro-2-furaldehyde-2-β-methyl semicarbazone incorporated in the paraffin blocks was increased to 1,000 μg, acid production was completely prevented in 17 (89%) of the 19 samples of saliva in which this concentration was used. A significant partial reduction in the amount of acid formed was noted in the remaining 2 samples.

Discussion. The evidence presented by this study indicates that the prevention of acid formation in saliva by 5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone and 5-nitro-2-furaldehyde-2-β-methyl semicarbazone is not adversely affected in the presence of the tissues and fluids of the oral cavity. In each instance a slightly higher concentration of these compounds was required when added *in vivo* than *in vitro* in order to inhibit acid pro-

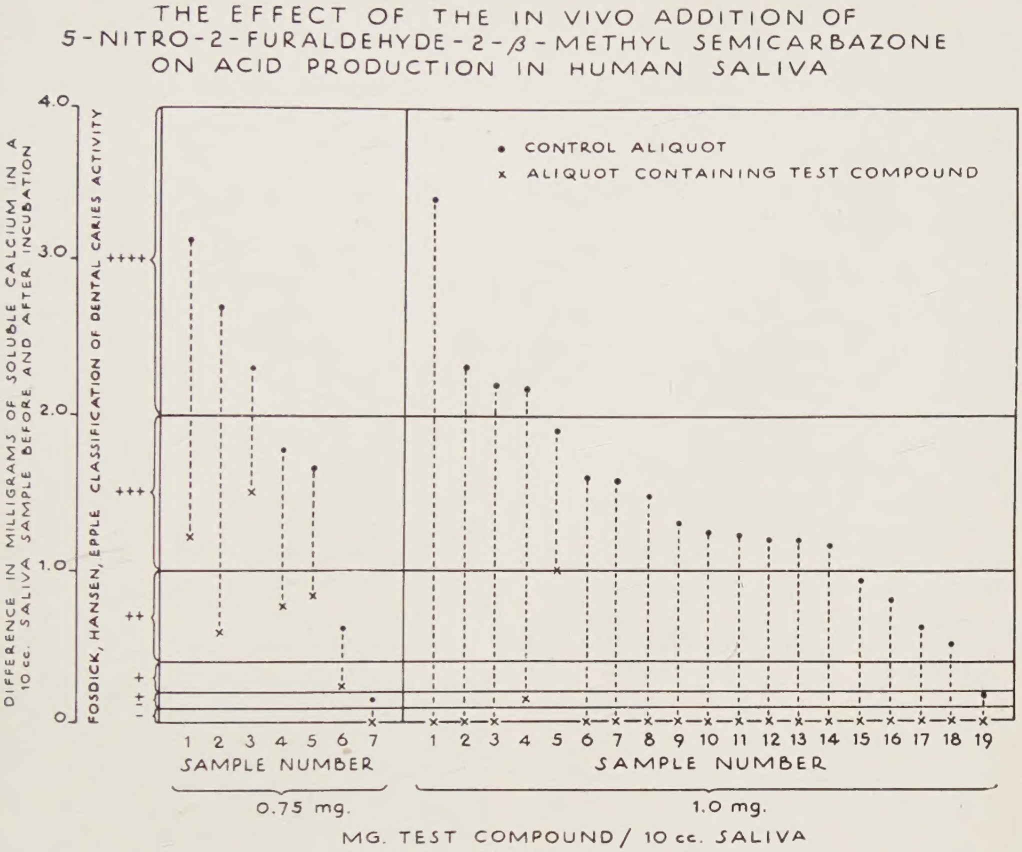

FIG. 3.

The sample numbers are arranged in the order of decreasing dental caries activity in the control aliquot and not in the order in which the samples were collected.

duction in the saliva of caries active patients. The difference may be attributed in part to the unavoidable loss of some of the test material by admixture with the paraffin used as the secretory stimulant, by swallowing, and by retention in the sheltered areas and crevices of the oral cavity.

Each of the test compounds is but slightly soluble in water, the solubility being 1:900 for 5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone, and 1:3000 for 5-nitro-2-furaldehyde-2-β-methyl semicarbazone. It appears, therefore, that the actual inhibitory activity of these compounds may be attributed to a fraction of the concentrations employed in this and in the previous study. The difference in the extent of the solubility of the test compounds may also account for the greater effectiveness of 5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone as an inhibitor of acid production in the saliva when added in 750 μg quantities *in vivo,* as compared with a similar amount of the less soluble 5-nitro-2-furaldehyde-2-β-methyl semicarbazone.

Summary and Conclusions. The incorporation of 1,000 μg quantities of 5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone and 5-nitro-2-furaldehyde-2-β-methyl semicarbazone in paraffin blocks used as salivary stimulants completely prevented acid production in 38 of the 42 saliva samples obtained from caries active individuals. A significant partial reduction in acid formation occurred in the remaining 4 cases. The findings indicate that 5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone and 5-nitro-2-furaldehyde-2-β-methyl semicarbazone are not rapidly destroyed or absorbed by the tissues and fluids of the oral cavity.

16993 P

Enhancement of Growth of Certain Fungi by Streptomycin.

CHARLOTTE C. CAMPBELL AND SAMUEL SASLAW. (Introduced by Maurice Landy.)
From the Department of Bacteriology, Army Medical Department Research and Graduate School, Army Medical Center, Washington, D.C.

Recent reports[1-7] have shown that the growth of some streptomycin-resistant bacteria may be dependent upon or enhanced by this antibiotic. A primary requisite for this phenomenon was previous exposure of these organisms to this drug. Although it is well known that streptomycin is not effective against fungi, there have been no observations, to our knowledge, concerning the enhancement of growth of fungi by streptomycin. The incorporation of streptomycin into certain selective media for fungi[8] has been instituted for the purpose of inhibition of bacteria. Employing a synthetic liquid medium containing streptomycin, we noted that the growth of certain fungi not previously exposed to streptomycin was more luxuriant than when streptomycin was absent. To further evaluate this observation, the studies described below were conducted on available laboratory strains of fungi which also had not been previously exposed to streptomycin.

Methods. The fungi were assayed in triplicate in a synthetic liquid medium consisting of inorganic salts and 0.5% glucose* to which commercial streptomycin† was added in concentrations of 0.01 to 5.0 mg/ml of medium. The final pH of the medium was 4.0. Inocula were prepared by washing the growth from 3-weeks-old Sabouraud's cultures with sterile saline and adjusting the turbidity to match a No. 3 MacFarland nephelometer standard. The suspensions were homogenized as much as possible by vigorous shaking and pipetting, and the heavier particulate matter allowed to settle. One-tenth ml amounts were then inoculated in triplicate into the test media as well as into control tubes without streptomycin. All cultures were incubated at 28°C and examined daily for comparative growth for 4 weeks.

Results. The growth of all of 6 strains of *Sporotrichum schenckii,* 4 of 4 strains of *Coccidioides immitis,* 2 of 3 of *Histoplasma*

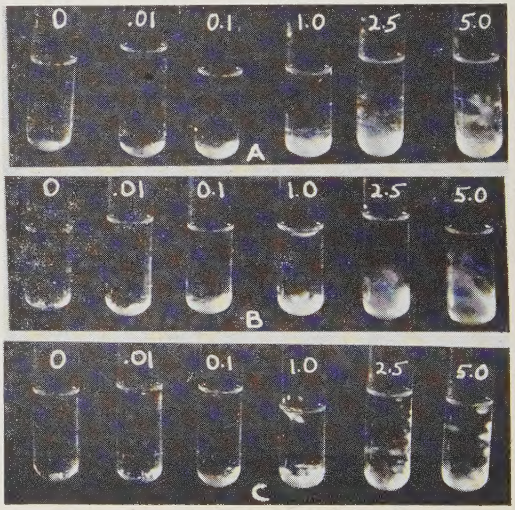

PLATE 1.

Effect of streptomycin on the growth of fungi. Each figure represents mg streptomycin/ml of medium.

A. *S. schenckii*—8th day.
B. *C. immitis*—9th day.
C. *H. capsulatum*—9th day.

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* 4.5 g KH_2PO_4, 0.5 g $(NH_4)_2SO_4$, 0.5 g $(NH_4)Cl$, 25 ml M/500 in N/50 HCl $FeNH_4SO_4$, 10 ml 0.4% $MgSO_4 \cdot 7H_2O$, 5.0 g glucose and distilled water to 1000 ml.

† Duplicate studies were done with Merck's streptomycin hydrochloride and Pfizer's streptomycin sulfate.

capsulatum as well as single strains of *Phialophora verrucosa* and *Trichophyton mentagrophytes* appeared to be increased in the presence of streptomycin. The stimulatory effect was noted as early as 48 hours with *S. schenckii* while similar results were obtained with the other organisms in periods varying from 1-3 weeks. The stimulatory effect was proportional to the concentration of streptomycin up to 2.5 mg per ml, at which level maximum growth was obtained. This effect was noted only with young actively growing cultures and not with old stored suspensions. Plate 1 depicts photographically the results obtained in representative tests.

Since streptomycin is known to be more effective at an alkaline pH, similar studies were made with *S. schenckii* and *C. immitis* in media adjusted to 4.0, 6.0, and 8.0, respectively. Although the amount of growth in the control tubes was considerably less at pH 8.0 than in the acid range, the same stimulatory effect in the presence of streptomycin noted above was observed at all 3 levels.

Summary. Streptomycin incorporated into a synthetic liquid medium enhanced the growth of laboratory strains of *S. schenckii, C. immitis, P. verrucosa, T. mentagrophytes,* and *H. capsulatum* which had not been previously exposed to this antibiotic.

16994

Phosphoprotein Phosphatase in Rat Young, Embryos, and Placentas.

MURRAY E. VOLK*† AND ROBERT N. FEINSTEIN. (Introduced by Kenneth P. DuBois.)
From the Toxicity Laboratory and the Department of Biochemistry,‡ University of Chicago.

Phosphoproteins appear to exist uniquely in food sources for the embryo and young (*e.g.*, casein in milk, vitellin in egg yolk, ichthulin in fish eggs). A clue to the significance of this fact was seen in the observation by Harris[1] that frog eggs contained an enzyme, designated phosphoprotein phosphatase, which released inorganic phosphorus from phospho-proteins without preliminary proteolysis. This was confirmed by Feinstein and Volk,[2] who further showed such an enzyme present also in various mammalian tissues.

The present report presents data regarding the activity of this enzyme in rat placentas, embryos, and young, all of various ages.

Methods. Dated conceptions were obtained by following vaginal smears and introducing the female to the male during an overnight period during estrus. At the desired time after conception, the pregnant female was sacrificed, and the embryos and placentas were removed, separated by rough dissection, quickly frozen in an ether-dry ice mixture, and stored under deep freeze until convenient for assay. Several placentas from each female were pooled for assay, as were 14- and 16-day embryos. Young rats were sacrificed by immersion in the freezing mixture and stored under deep freeze.

For assay, placentas and embryos were homogenized in a glass homogenizer; young rats were blended in a Waring blendor. Further details of the assay and analytical methods have been described.[2]

Results. Fig. 1 presents the phosphoprotein phosphatase activity of whole placentas, embryos, and young rats, all of varying ages.

Fig. 2 presents the activity of this enzyme per gram of tissue and per mg of protein, in

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† Present address: Research Institute, Temple University, Philadelphia, Pa.

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[1] Harris, D. L., *J. Biol. Chem.*, 1946, **165**, 541.

[2] Feinstein, R. N., and Volk, M. E., *J. Biol. Chem.*, in press.

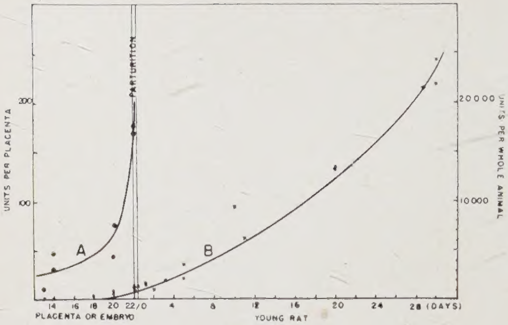

FIG. 1.

Age of (A) placenta and (B) whole animal, vs. total phosphoprotein phosphatase activity.

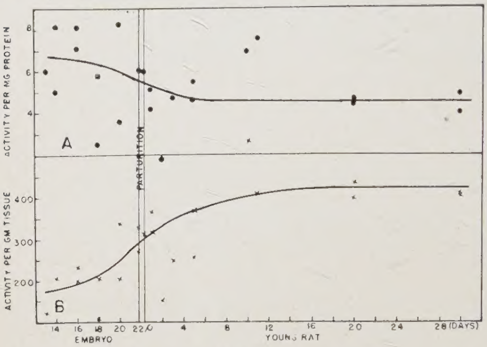

FIG. 2.

Age of animal vs. phosphoprotein phosphatase activity (A) per mg of protein and (B) per gram of body weight.

relation to the age of the embryo or young rat. Curve A, concerning activity per mg of protein, has too high a degree of scattering in the early points to warrant positive views as to the exact shape of the curve. The excessive scattering is believed due to unsatisfactory protein determinations, since the method used, that of Robinson and Hogden,[3] yielded unclear color solutions in some cases.

Fig. 3 relates placental age to enzyme activity per g tissue and per mg protein. The comment of the preceding paragraph, regarding the protein determination, is also relevant here.

Discussion. The results, particularly those of Curve B, Fig. 2, are approximately those to be expected from teleological considerations. The activity in the embryo is seen to rise, slowly at first, then more rapidly as parturition is reached. Thus the newborn

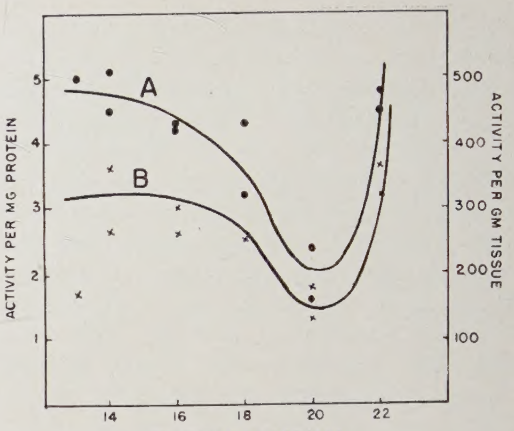

FIG. 3.

Age of placenta vs. phosphoprotin phosphatase activity (A) per gram of tissue and (B) per mg of protein.

animal, suddenly provided with phosphoprotein (casein) as a basic food source, is simultaneously equipped to utilize it. As the animal continually increases his casein intake, the enzyme activity continues to rise. At approximately 12 days of age, the enzyme activity reaches a constant level, which is unaffected by weaning.

Summary. 1. A study has been made of the level of phosphoprotein phosphatase activity in placentas, whole embryos, and whole young growing rats of various ages.

2. The total phosphoprotein phosphatase activity of the placenta and of the embryo and young rats continually rises. The activity was followed in placenta and embryo from thirteen days post-conception to parturition, and in young rats to 28 days of age.

3. The activity per gram of body weight rises slowly in early embryos, rapidly in the period immediately preceding and following parturition, and remains essentially constant during the age period of 12 to 28 days.

4. The activity per gram of placenta decreases from the 14th to the 20th day after conception, then rises again just before parturition. The preliminary decrease is due to the rate of weight gain of the placenta being greater than the rate of gain of total placenta phosphoprotein phosphatase; the reversal of direction of the curve is due to continued enzyme increase while the placenta gains essentially no further weight.

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[3] Robinson, H. W., and Hogden, C. G., *J. Biol. Chem.*, 1940, **135**, 707, 727.